STUDIES ON STRESS RESPONSE OF FISH CELLS(Dissertation_全文)

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To my family and to my wife Yasuko

ABBREVIATIONS

ATP	adenosine 5'-triphosphate
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EDTA	ethylendiaminetetraacetic acid
EGTA	ethylene glycol bis (β-aminoethylether)-*N, N, N', N'*-tetraacetic acid
ERK	extracellular signal-regulated kinase
GST	glutathione S-transferase
HA	hemagglutinin
HOG1	high osmolarity glycerol response 1
IgG	immunoglobulin G
IL-1	interleukin-1
JNK	c-Jun N-terminal kinase
kb(p)	kilobase (pair)
kDa	kilodalton
LPS	lipopolysaccharide
MAP kinase	mitogen-activated protein kinase
MAPKK	MAP kinase kinase
MAPKKK	MAP kinase kinase kinase
mRNA	messenger RNA
NES	nuclear export signal
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT	reverse transcription
SAPK	stress-activated protein kinase
SDS	sodium dodecyl sulfate
SSC	standard sodium chloride/sodium citrate buffer
TNF	tumor necrosis factor
Tris	tris (hydroxymethyl) aminomethane
UTR	untranslated region
UV	ultraviolet

CONTENTS

INTRODUCTION

In the natural environment, although fish generally spawns a large number of eggs at a time, very few will be fortunate enough to grow up to adults. This is believed to be caused by high mortality at the early developmental stage including embryonic stage. There is a fair chance for fish embryo to encounter environmental stresses such as osmotic stress, heat shock, UV light, and water pollutants, because of its ectogenetic early development. However, fish embryo is not yet equipped with the highly organized stress-responsive system such as an endocrinological and a neurological regulations. Lack of this system in the embryo might lead, at least in part, to the early mortality. On the other hand, it is assumed that fish embryo possesses some mechanism of survival strategy at a cellular level, for some proportion of the population survives against exposure to environmental stresses.[1] To date, stress response of fish cell retains a lot to be elucidated. In this study, the author uses fish cell cultures *in vitro* as a simplified model of fish cells *in vivo*.

In Chapter I, the author shows that intrinsic viabilities of fish cell lines including EPC (a carp epithelial cell line, *Epithelioma Papulosum Cyprini* [2]) to osmotic stress are different from those of mammalian cell lines. First, the author examined the cell growth of various fish cell lines in hyper- and hypotonic environments in comparison with several mammalian cell lines. Because EPC exhibited a typical pattern of cell growth among the fish cell lines in the hyper- and hypotonic environments, apoptotic cell death of EPC was also examined in detail. Apoptosis is an active mode of cell death which requires gene expression induced by environmental stress or certain cytokines.[3-7] Thus, the cellular response of EPC can be estimated by the extent of apoptosis in the stress-exposed cell culture as well as the growth rate. By evaluating cell growth and cell death in hypertonic environment, the author succeeded in demonstrating that EPC adapts and acclimates to hypertonic environment. For further study, the author applies a carp epithelial cell line EPC to investigate the mechanism of stress response.

An important part of the cellular response to a changing environment is to adjust the levels of gene products to new conditions. To initiate these adaptations, signals have to be relayed from the plasma membrane to the nucleus. The JNK/SAPK (cJun N-terminal kinase/stress-

activated protein kinase) and the p38 MAP (mitogen-activated protein) kinase pathways have been recently identified in higher vertebrates as signal transduction pathways to transmit the signals including osmotic stress by phosphorylation through a protein kinase cascade.[8-16] These two pathways as well as the classical MAP kinase pathway, which is the first identified pathway for transduction of growth and differentiation signals,[17-22] consist of three protein kinases forming the protein kinase cascade. In the classical MAP kinase pathway, MAPKKK (MAP kinase kinase kinase) phosphorylates and thereby activates MAPKK (MAP kinase kinase), and the activated form of MAPKK in turn phosphorylates and activates MAP kinase. The activated MAP kinase may translocate to the cell nucleus and regulate the activities of transcription factors and thereby control gene expression. The JNK/SAPK and the p38 MAP kinase pathways have been shown to function in a similar manner in response to environmental stresses.[8-16]

Again, the signal transduction from the plasma membrane to the nucleus is the primary response to environmental stress, which in turn induces the secondary response, that is, gene expression for adaptation or apoptotic cell death.[23, 24] Therefore, the cell should prepare the signal transduction pathways to respond environmental stresses which it might encounter in the future. It is important to elucidate the mechanism of the primary response in fish cells, since there is a considerable number of chances to be exposed to environmental stresses.

In Chapter 2 and Chapter 3, the author identifies and characterizes the JNK/SAPK and the p38 pathways in carp, respectively, in order to know whether these pathways respond rapidly to environmental stress including hypertonic stress. The results shown in these chapters suggest that there may be a functional difference between these two stress-responsive pathways of carp and also that the regulation of the p38 MAP kinase pathway may be more precise than that of other higher vertebrates.

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CHAPTER I

Cell Growth and Apoptosis under Hypertonic Condition

Section 1
Comparison of Cell Growth between Fish and Mammalian Cell Lines

There are many reports on the endocrinological mechanism of osmotic regulation in fish.[1] However, it is poorly known about cellular response of fish cells to various osmotic environments. In this study, the author investigated the growth of fish cell lines, EPC (carp epithelioma origin),[2] EK-1 (eel kidney origin),[3] BF-2 (bluegill caudal trunk origin),[4] and RBCF-1 (goldfish fin origin),[5] in hyper- and hypotonic media in order to clarify the susceptibilities of fish cells to hyper- and hypoosmolarities. Mammalian cell lines, HeLa (human carcinoma of cervix origin), NIH/3T3 (mouse embryo origin), BALB/3T3 (mouse embryo origin), and L6 (rat muscle origin), were also employed for comparison.

Materials and Methods

Media

The hyper- and hypotonic media were prepared by doubling and halving the concentration of the inorganic salts in the medium. Eagle's minimum essential medium (MEM, Nissui) was used for HeLa, NIH/3T3, BALB/3T3, L6, EPC, and BF-2. For EK-1 and RBCF-1, Leibovitz L-15 medium (Gibco-BRL) was employed. Osmotic pressures of hypo-, iso-, and hypertonic media (both MEM and Leibovitz L15) were approximately 200-210, 300-310, and 470-480 mOsm/kg, respectively.

Measurement of Cell Growth

Cells were plated in the isotonic medium containing 10 % fetal bovine serum (FBS) at the density of about 2.0×10^5 cells per dish (Φ 35mm), and pre-cultured for a day: HeLa, NIH/3T3, BALB/3T3, L6, BF-2, EK-1, and RBCF-1. EPC was inoculated at the density of 1.0×10^6 cells per dish because of difference in cell size. Cell growth was measured by counting the cell number per dish according to the method described previously.[6]

DNA Fragmentation Assay

EPC was plated and cultured in the same manner described in *Measurement of Cell Growth*. On day 1 the medium was changed to the hypo- (a), iso- (b), or hyper- (c) tonic medium containing 10% FBS, and at 0 h (cont.), 3 h, 24 h, 48 h, 96 h, and 144 h after this treatment (0 h, 48 h, 96 h, and 144 h are corresponding to 1, 3, 5, and 7 culture days in Fig. 1-1) the cells were harvested in lysis buffer (10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5 % Triton X-100). The culture without any treatment was also harvested on day 7 (cont. 144 h). After centrifugation ($1,3000\times g$, 20 min) of the cell lysates, the supernatants were treated subsequently with RNase A (0.4 mg/ml, Sigma) and Proteinase K (0.4 mg/ml, Nacalai tesque). The precipitations were retreated in a solution containing 0.5 % SDS, 10 mM Tris-HCl (pH 8.0), and 10 mM EDTA to solubilize the genomic DNA from the native nucleus of the cells that did not undergo apoptosis. The samples of the supernatant (sup) and the precipitation (ppt) were electrophoresed on 1.6 % agarose gels to see DNA ladders and the concentrations of the cells in the samples (7×10^5 cells/lane).

Results and Discussion

Figure 1-1 shows the growth curves of each cell line during 7-days culture. For all the cell lines, the highest growth was observed in the isotonic medium. Fish cell lines generally exhibited higher growth in the hypotonic medium than in the hypertonic medium except for RBCF-1. In contrast, all the mammalian cell lines showed the better growth rates in the hypertonic medium than in the hypotonic medium. This feature of the mammalian cell lines was most remarkable in NIH/3T3, whose cultures underwent cell death of the whole population within 1 day after starting the culture in the hypotonic medium. Interestingly, although growth inhibition or cell death was seen in EPC and EK-1 during the first 2 days in the hypertonic medium, the growth rate was recovered afterwards. In consistent with these results, the author observed by detecting DNA ladders, which are defined as a hallmark of apoptosis,[7] that EPC underwent apoptotic cell death within 3h

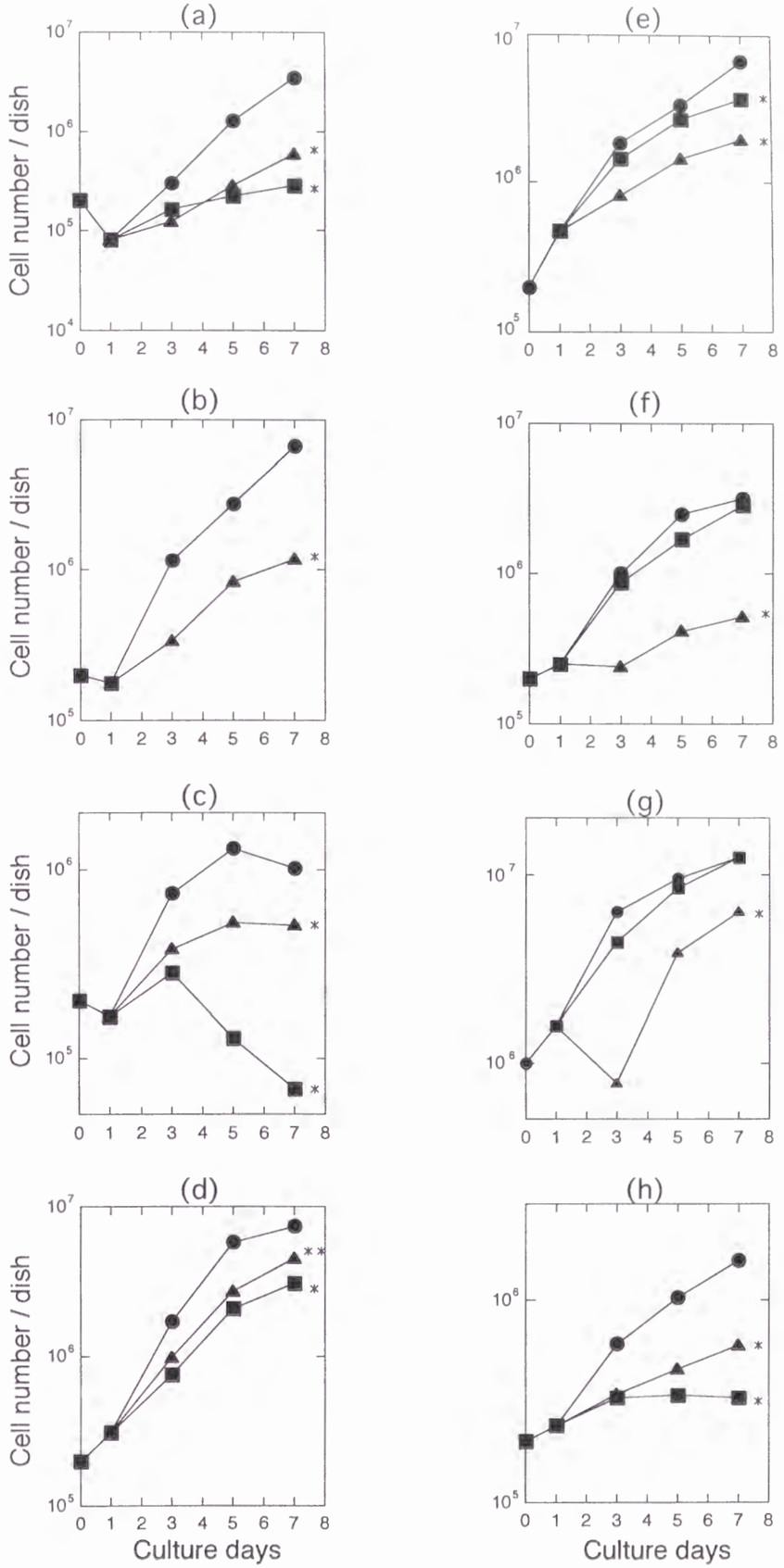
after replacing the medium to the hypertonic medium although DNA ladder was significantly decreased after a longer span (1-6 days) of culture (Fig. 1-2). These findings suggest that these cell lines acclimated to hypertonic environments within the first few days of culture, and furthermore, that some portion of EPC cells not resistant against hypertonic stress underwent cell death by apoptosis not by necrosis within a few hours after exposure to the hypertonic medium probably for the effective elimination of these cells. The growth of BF-2 cells was not affected even in the hypertonic medium (Fig. 1-1).

In this study, no common feature of growth response in different osmotic media was seen in the cell lines derived from the euryhaline (BF-2 and EK-1) and the stenohaline (EPC and RBCF-1). This fact is consistent with the report by Fernandez *et al.* who observed no common response of growth among the cell lines from salmonids and non-salmonids in various osmotic media.[8)]

In conclusion, the present investigation revealed that EPC, EK-1, and BF-2 grew at a high rate in the hypotonic medium and that EPC and EK-1 seemed to acclimate to the hypertonic medium. These features of the fish cell lines were not observed in mammalian cell lines examined in this study. The high adaptability of the fish cell lines, BF-2, EK-1, and EPC, to different osmolarities raises a requirement for a future study on the osmotic-responsive mechanism in fish cells.

Fig. 1-1. Cell Growth of Fish and Mammalian Cell Lines in Hyper- and Hypotonic Media.

Cells were plated in the isotonic medium containing 10 % fetal bovine serum (FBS): (a) HeLa, (b) NIH/3T3, (c) BALB/3T3, (d) L6, (e) BF-2, (f) EK-1, (g) EPC and (h) RBCF-1. On day 1 the medium was changed to the hyper- (▲), hypo- (■), or iso- (●) tonic medium containing 10% FBS. The number of cells was determined by counting cells of three independent dishes on the day indicated. Since all the population of NIH/3T3 cells died within 2 days, no sign of the cell number in the hypotonic medium (■) afterwards is shown in the figure. The decrease in the cell number of HeLa (a), NIH/3T3 (b), and BALB/3T3 (c) in the initial day of culture was due to the failure of some portion of the cells in attaching to the dish. Significant differences between the isotonic medium and the hyper- or hypotonic medium determined on day 7 by the paired Student's *t*-test are shown as stars (*: $p<0.005$, **: $p<0.01$).



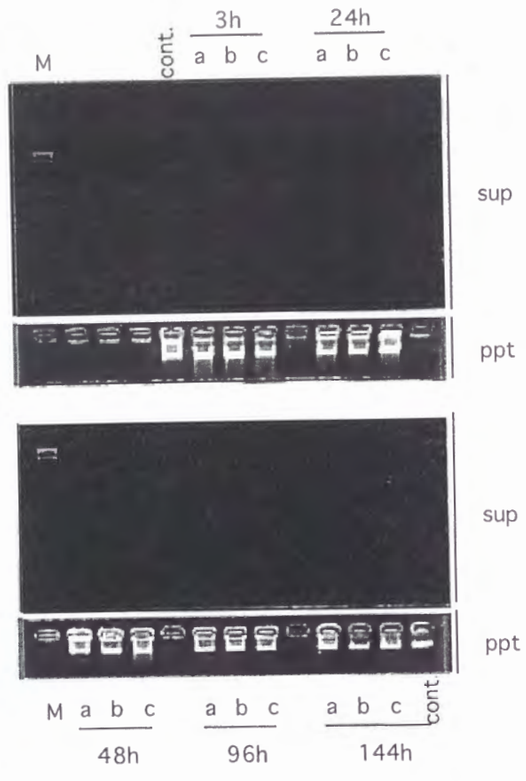


Fig. 1-2. Apoptotic Cell Death of EPC.

EPC was plated and cultured in the same manner described in the legend of Fig. 1-1. On day 1 the medium was changed to the hypo- (a), iso- (b), or hyper- (c) tonic medium, and at 0 h (cont.), 3 h, 24 h, 48 h, 96 h, and 144 h after this treatment (0 h, 48 h, 96 h, and 144 h are corresponding to 1, 3, 5, and 7 culture days in Fig. 1-1) the cells were harvested. The culture without any treatment was also harvested on day 7 (cont. 144 h). Fragmented DNA (sup) and the native DNA (ppt) were electrophoresed on 1.6 % agarose gels to see DNA ladders and the concentrations of the cells in the samples (7×10^5 cells/lane). Note that approximately the same amounts of genomic DNA were detected in all lanes, which supports that each sample electrophoresed in a lane was derived from the same number of cells. "M" stands for DNA marker (*Hin*dIII-digested λDNA).

Section 2
Apoptotic Cell Death of EPC Induced by Hyperosmotic Stress

Apoptosis is a physiological and pathological mode of cell death in an active and inherently controlled manner.[9-10] It occurs in normal processes of development and differentiation,[11-16] or in response to moderate damaging stimuli such as environmental stress and noxious agents.[17-20] In contrast to necrosis, cell death by apoptosis is accompanied by some morphological changes including and condensation of nucleous and cytoplasm and thier budding into multiple apoptotic bodies.[9] Biochemical features of apoptosis are characterized by a well-defined phenomenon of DNA fragmentation resulting from the digestion of chromatin at the internucleosomal linker regions. The DNA fragmentation events can be observed as a typical ladder pattern on agarose gel electrophoresis, and are defined as a biochemical hallmark of apoptosis.[7]

Cell death by apoptosis seems to be physiologically meaningful in the sense that it avoids an inflammatory response by preventing intracellular materials from leaking.[21] Osmotic change is one of the environmental stresses and can directly cause the leakage of intracellular material by cell shrinking or swelling.

Fish cells seem to be a good model for the physiological studies on cellular response to osmotic stress, because there is a considerable number of chances for fish, thereby encountering a variety of osmotic pressure for fish cells particularly epithelial cells like EPC. Thus, they are likely to be equipped with highly advanced system for responding to osmotic stress. In fact, the author previously reported that several fish cell lines exhibited a different growth pattern in hyper- and hypotonic environments from mammalian cell lines.[22] To date, however, cell death as an environmental response of fish cells heve not been well characterized.

In this study, the author investigated the effects of a wide range of osmotic pressure on the DNA fragmentation using the carp epithelial cell line EPC, which originated from a skin hyperplastic lesion.[2]

Materials and Methods

Media

EPC was subcultured at 30℃ in air in Eagle's minimum essential medium (MEM, Nissui) supplemented with 10% fetal bovine serum (FBS, Biological Industries) and 2.5 mM HEPES (*4*-(*2*-hydroxyethyl)-*1*-piperazineethanesulfonic acid), whose osmotic pressure was 300 mOsm/kg. A wide range of different osmotic media (150-600 mOsm/kg) was prepared with 50-fold concentrated MEM amino acid solution (Gibco-BRL), 100-fold concentrated MEM vitamin solution (Gibco-BRL), and the other components except sodium chloride supplied to the same final concentration as the standard MEM. Only the concentration of sodium chloride was different among the various osmotic media. To give 450 mOsm/kg with sorbitol, an appropriate amount of sorbitol was added to the 300 mOsm/kg medium prepared as described above. Osmotic pressure of all the media and phosphate-buffered saline (PBS) was measured with Osmometer OM-801 (Vogel).

Exposure of EPC to Various Stresses

EPC was inoculated in a 60-mm plastic dish (Nunc) in MEM supplemented with 10% FBS at a density of 2×10^6 cell per dish and cultured for 3 days. After replacing the medium with 300 mOsm/kg medium, the cells were pre-cultured for 15 h. Then they were exposed to various stresses as follows: Osmotic stresses were given by exchanging the medium to the one with different osmotic pressure. Heat shock and cold shock were given by placing the dishes in 40℃ and 20℃ water baths, respectively. Actinomycin D (Sigma) treatment was done by adding 1 mg/ml solution to the medium at a final concentration of 2 μg/ml. In the case of UV irradiation, the medium was temporarily removed and the cells were exposed to UV light at a dose of 80 J/m^2.

DNA Fragmentation Assay

The extent of DNA fragmentation was examined according to the method of Sellins and Cohen[23] with a slight modification. At various times after exposure to stresses, all the cells dead or alive were harvested by mechanically detaching and centrifugation at $200\times g$ for 5 min. The pellet was lysed on ice with 100 μl lysis buffer (10 mM Tris-HCl, 10 mM EDTA, pH 7.5) containing 0.5% Triton X-100. The lysate was centrifuged at $13,000\times g$ for 20 min.

The resulting supernatant, containing solubilized DNA fragments ("sup" in Fig. 1-4 and all the samples subjected to electrophoresis in other Figs.), was placed in a separate tube, and after treatments of Proteinase K (Nacalai Tesque) for 1 h and RNase A (Sigma) for 1 h at 37℃, it was precipitated at -20℃ in 5% sodium chloride and 50% *2*-propanol. The precipitates were collected by centrifugation at $13,000\times g$ for 15 min.

The insoluble DNA after cell lysis ("ppt" in Fig. 1-4) was treated with 250 μl of the lysis buffer containing 0.5% SDS and homogenized by passing through a 23G needle.

Each of one sixth of the supernatant and one twenty-fifth of the insoluble pellet was electrophoresed on a 1.7% agarose gel. All the samples electrophoresed on one agarose gel were derived in a single series of experiment. DNA marker consists of *Hin*dIII-digested λDNA and a 1137 bp DNA fragment.

Fluorescence Microscopy with Hoechst 33258 Staining

Cells were plated and pre-incubated under the same condition except for the use of 24-well plate. After exposing the cells to various osmotic stresses, the culture medium was removed and the cells were fixed by PBS containing 1% glutaraldehyde. DNA staining was done in PBS containing 1 mM Hoechst 33258 (Nacalai Tesque) at room temperature for 30 min. Apoptotic nuclear morphology was observed in the fluorescence microscope (Olympus) with UV/blue filter (λ400-440).

Inhibition of DNA Fragmentation

$ZnCl_2$ was added to the medium at a final concentration of 1 mM at 1 h before the exposure to stresses. The cells were exposed to stresses in the presence of 1 mM $ZnCl_2$.

Results

DNA Fragmentation in EPC

Several environmental stresses and treatment of actinomycin D, which are known to induce cell death by apoptosis in mammalian cells,[18-20, 24, 25] were given to EPC to know whether the DNA fragmentation assay was

available for detecting apoptosis in EPC. Cold shock (10℃ lower than usual culturing temperature of 30℃) was also given to compare with heat shock (10℃ higher). As a result, DNA fragmentation was observed as a typical apoptotic DNA ladder appearing in several bands of 180 bp-multimers in agarose gel electrophoresis when the cells were exposed to UV irradiation, heat shock, and actinomycin D (Fig. 1-3). More significant DNA fragmentation was observed at 3 h rather than at 1 h after induction by these stresses. Faint DNA ladders were detected after serum-removal or exposure to cold shock, but no significant increase in DNA fragmentation after these treatments was observed.

Effects of Osmotic Pressure on DNA Fragmentation in EPC

To prepare various osmotic media, the concentration of sodium chloride in MEM was decreased or increased to give each osmotic pressure (150-600 mOsm/kg, see *Media* in Materials and Methods). When EPC was exposed to hypertonic (450 mOsm/kg) medium, the extent of DNA fragmentation was significantly increased in comparison with the case of 300 mOsm/kg (Fig. 1-3). Hypotonic (150 mOsm/kg) medium did not induce any significant DNA fragmentation (Fig. 1-3).

To investigate in detail the effects of osmotic pressure as an inducer of apoptosis, the author tested for the DNA fragmentation in 150, 200, 300, 350, 450, and 600 mOsm/kg media. The assay showed that treatments with 150-350 mOsm/kg media did not trigger apoptosis within 3 h (Fig. 1-4). The same result was obtained in 150 and 450 mOsm/kg media (Figs. 1-3 and 1-4).

In order to assess the accuracy of the DNA fragmentation assay and to estimate the population of the cells which did not undergo apoptosis, the insoluble fraction of the cell lysate which contained non-fragmented genomic DNA of living cells was solubilized by SDS treatment and electrophoresed on an agarose gel (Fig. 1-4). Approximately an equal amount of genomic DNA was observed for each medium with various osmotic pressures (150-450 mOsm/kg), suggesting that most of the population did not undergo apoptotic cell death, even in 450 mOsm/kg medium where significant DNA fragmentation was observed. However, the amount of genomic DNA was markedly decreased when treated with 600 mOsm/kg medium.

Cell death by apoptosis is known to be generally accompanied by

morphological changes due to nuclear condensation or generation of apoptotic bodies.[20] To know whether cell death of EPC triggered by osmotic stress exhibits condensed nuclei, nuclear morphology was assessed by fluorescence microscopy as for EPC treated with 150, 300, and 450 mOsm/kg media for 3 h. A large number of condensed nuclei stained by DNA-specific dye Hoechst 33258 were observed in 450 mOsm/kg, while very few were seen in 150 and 300 mOsm/kg (Fig. 1-5). Smaller but significant number of condensed nuclei were observed in 400 mOsm/kg medium (data not shown). Large nuclei dimly luminous on the background of each microphotograph indicate the population of the cells which were alive and attached on the dish (Fig. 1-5). These data confirm that the treatment with 450 mOsm/kg medium brought about cell death by apoptosis in a small portion of the population in EPC culture.

In order to know whether longer treatment with hypertonic media increases the incidence of DNA fragmentation, incubation time was extended to 9 h (Fig. 1-6). In 400 and 450 mOsm/kg, increasing amounts of fragmented DNA were observed from 1 h to 3 h after the osmotic changes. But no further increases were seen in both media. No significant DNA fragmentation was detected in 350mOsm/kg medium.

Severer stresses were given with 500 and 600 mOsm/kg media and the incidences of apoptotic cell death were examined by DNA fragmentation assay (Fig. 1-6). The amounts of fragmented DNA increased during the first 3 h after the osmotic changes in 500 mOsm/kg. Interestingly, less DNA fragmentation was observed in higher osmotic medium, 600 mOsm/kg. In order to evaluate in detail cell death in 600 mOsm/kg, trypan blue staining was done to assess cell death at 3 h after exposure. Approximately 65% of the population in 600 mOsm/kg medium was stained while less than 5% of the cells in 150-500 mOsm/kg media were stained (data not shown). These results suggest that EPC died by necrosis as well as by apoptosis in the osmotic pressure of 600 mOsm/kg.

Effect of Hyperosmolarity by Sorbitol Supplementation on Apoptosis

In order to know whether apoptosis triggered by hyperosmolarity is influenced by difference in ionic activity of the osmolytes, the author investigated the effect of sorbitol on cell death of EPC, using 450 mOsm/kg medium with sorbitol. DNA fragmentation was examined in comparison

with 450 mOsm/kg with sodium chloride. Addition of sorbitol to the medium induced to a similar extent to the case of sodium chloride apoptotic cell death in both microscopic observation (Fig. 1-5) and DNA fragmentation assay (Fig. 1-7).

Inhibition of DNA Fragmentation by Zn^{2+}

Several endogenous DNases have been reported to be involved in the executing step of apoptosis by cleaving nucleosomal DNA.[27-33] In EPC, the DNA fragmentation induced by hypertonic stresses (both 450 and 500 mOsm/kg) as well as by heat shock, UV irradiation, and actinomycin D was partially suppressed by 1 mM Zn^{2+} ion (Fig. 1-7).

Discussion

In the present study, the author demonstrated the suitability of the DNA fragmentation assay for investigating the response of a fish cell line, EPC to various environmental stresses. By employing this assay, UV irradiation and heat shock, which are known as triggers for apoptosis in mammalian cells, were also shown to be inducers for apoptosis in EPC (Figs. 1-3 and 1-4). In addition, actinomycin D was shown to be a noxious agent to induce DNA fragmentation in EPC (Figs. 1-3 and 1-4), although it inhibited apoptosis in some kinds of mammalian cells.[23, 34] Serum-removal did not induce apoptosis in EPC, while it often causes cell death by apoptosis in mammalian cells.[10, 25]

Small amounts of fragmented DNA were detected in the culture without exposure to stresses or with the isotonic medium (300 mOsm/kg) in this study (Figs. 1-3, 1-4, 1-6, 1-7, and 1-8). Maeda *et al.*[35] reported that high-cell-density caused apoptotic cell death in the isotonic medium. However, the cell-density of the 300 mOsm/kg cultures described above was less than 2×10^5 cells/cm^2. This value was by one-fifteenth fold lower than that in the plateau phase for EPC which has been reported to be at a density of 3×10^6 cells/cm^2.[2] Therefore, the constant DNA fragmentation in 300 mOsm/kg could more likely be caused by physiological turn-over in the population of EPC culture rather than by high-cell-density, which occurred without any stress.

In EPC, DNA fragmentation and apoptotic morphology, the signs

known as features of apoptotic cells, were not observed in hypotonic environment (Figs. 1-3, 1-4, and 1-6), nor were the signs of necrosis. In contrast, mammalian cell lines, HeLa and NIH/3T3 underwent apoptosis and necrosis by treatment with 150 mOsm/kg medium in his experiment, respectively (data not shown). A likely explanation for these differences in the response to hypotonic stress can be made by assuming that EPC should intrinsically be equipped with hypotonic responsive mechanism, because EPC was established from the skin of a freshwater fish, carp.

On the other hand, to be of much interest, hypertonic environment turned out to be an inducer of apoptosis in EPC. Remarkable increase in DNA fragmentation was observed within 3 h after exposure to hypertonic media of 400-600 mOsm/kg (Fig. 1-6). According to the results of trypan blue staining, under 600 mOsm/kg there was a large portion of the cell population undergoing necrosis, while only a trace of necrosis was seen under lower osmotic pressures. This is consistent with the result that decreased amount of genomic DNA was detected in the insoluble precipitate of cell lysate in 600 mOsm/kg (Fig. 1-4). These results suggest that between 500 and 600 mOsm/kg is a critical range for most of the population of EPC to choose the pathway of cell death by necrosis rather than by apoptosis.

A large proportion of the population in the cultures of EPC exposed to hypertonic stress (below 500 mOsm/kg) was capable of avoiding cell death by apoptosis or necrosis, which was assessed by the evaluation of amount of native nucleosomal DNA (Fig. 1-4) and the microscopic observation of the native nuclei (Fig. 1-5). Furthermore, the extent of DNA fragmentation in a small proportion of the population of the cultures increased only within the first 3 h after exposure to the hypertonic stress. Here, the author proposes that only a small proportion of the whole cell population is susceptible to hypertonic-induced apoptosis and finishes undergoing apoptotic cell death by the first 3 h in the hypertonic environment.

Among many candidates responsible for cleavage of nucleosomal DNA in apoptosis, Zn^{2+} susceptible endonucleases including DNase γ,[33] NUC18,[28] and Ca^{2+}/Mg^{2+}-dependent endonuclease[31] are most probable participants in the apoptotic pathway, because DNA fragmentation was inhibited in the presence of Zn^{2+} *in vivo*.[36] In this study, it was shown that hypertonic-induced DNA fragmentation was partially suppressed in the presence of Zn^{2+} (Fig. 1-7), suggesting that Zn^{2+} susceptible endonuclease

was involved in apoptosis under hypertonic condition in fish cells as well.

Among the studies on apoptosis in mammalian cells, there is only one report which shows cell death by apoptosis in human salivary gland cells (HSG) under hypertonic stress.[37] According to this report, HSG underwent apoptosis in sorbitol-added medium, while it died by necrosis in sodium chloride-added medium even in the same osmotic pressure. In this study, significant difference was not seen between sorbitol and sodium chloride in inducing apoptosis in EPC (Fig. 1-7). These imply the difference between HSG and EPC in susceptibility to high concentration of Na^{2+} ion.

In conclusion, his results indicate that a small proportion of the whole cell population undergoes apoptosis within a short term in the hypertonic environment. It should be elucidated in the future why the other proportion of the population is resistant to the hypertonic environment.

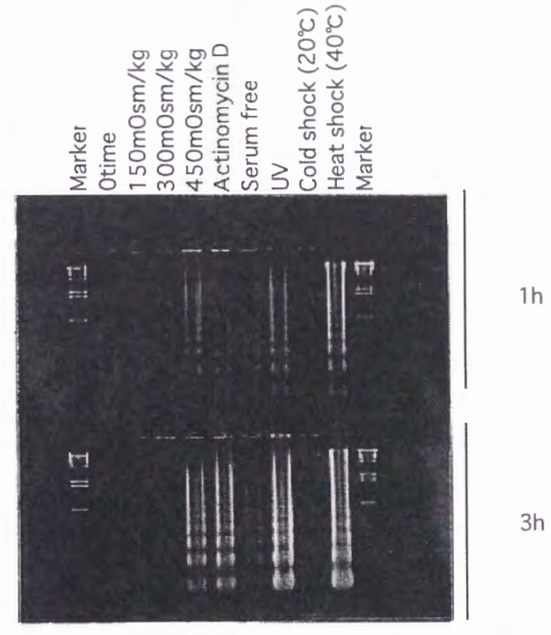


Fig. 1-3 DNA Fragmentation in EPC.

DNA fragmentation was examined in several cultures of EPC at 1 h and 3 h after exposure to 150, 300 (control), and 450 mOsm/kg media, actinomycin D (2 μg/ml), UV irradiation (80 J/m²), cold shock (20℃), heat shock (40℃), and serum-removal. The culture before exposure is shown as 0 time.

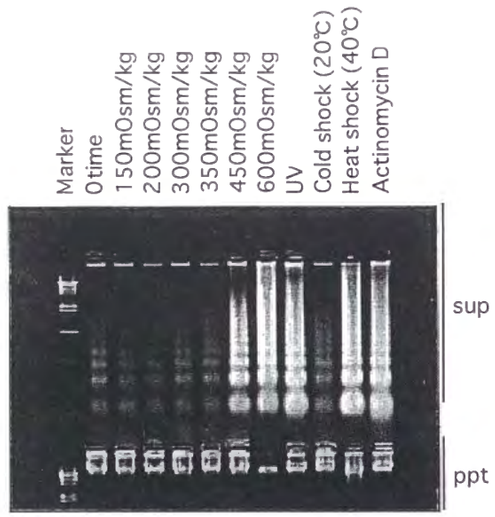


Fig. 1-4 Assessment of the Accuracy of the DNA Fragmentation Assay.

The solubilized DNA fragment fraction (above) and the insoluble precipitate (below) of cell lysate of the cultures exposed to stresses for 3 h (see *DNA Fragmentation Assay* in Materials and Methods) were electrophoresed on an agarose gel. The culture before exposure is shown as 0 time.

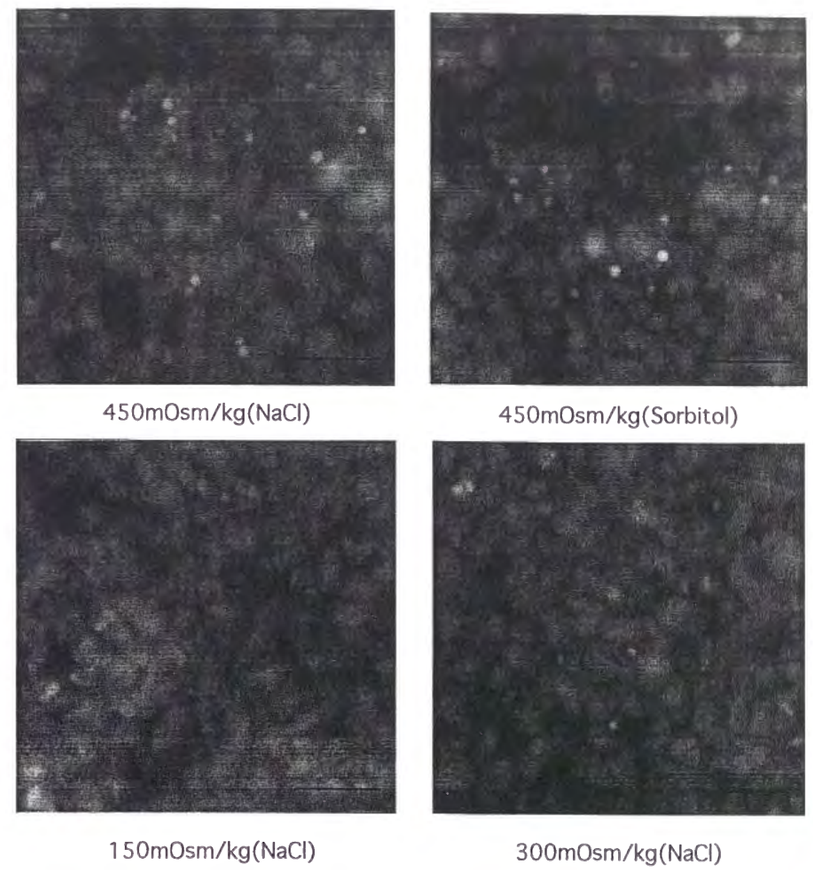
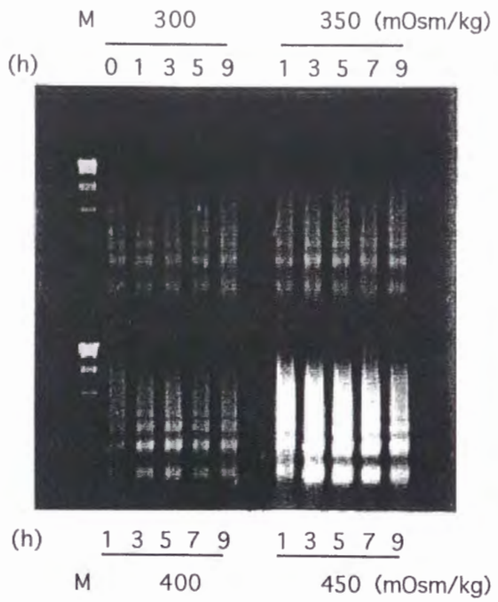


Fig. 1-5 Fluorescence Microscopy with Hoechst 33258 Staining.

The bars represent 50 μm.



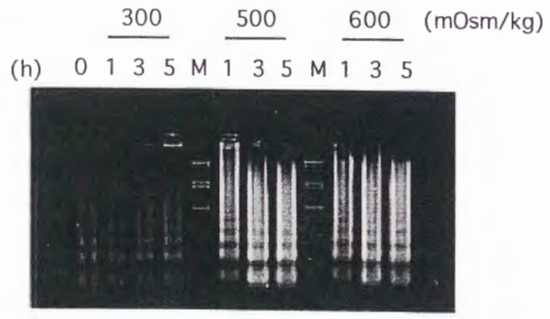


Fig. 1-6

(upper) Time Course of DNA Fragmentation in EPC.

DNA fragmentation was examined 1 h, 3 h, 5 h, 7 h, and 9 h after exposure to 300 (control), 350, 400 and 450 mOsm/kg media. The culture before exposure is shown as 0 h. 'M' stands for DNA marker.

(lower) Response to Severer Hypertonic Stress

DNA fragmentation was examined 1 h, 3 h, and 5 h after exposure to 300 (control), 350, 500 and 600 mOsm/kg media. The culture before exposure is shown as 0 h. 'M' stands for DNA marker.

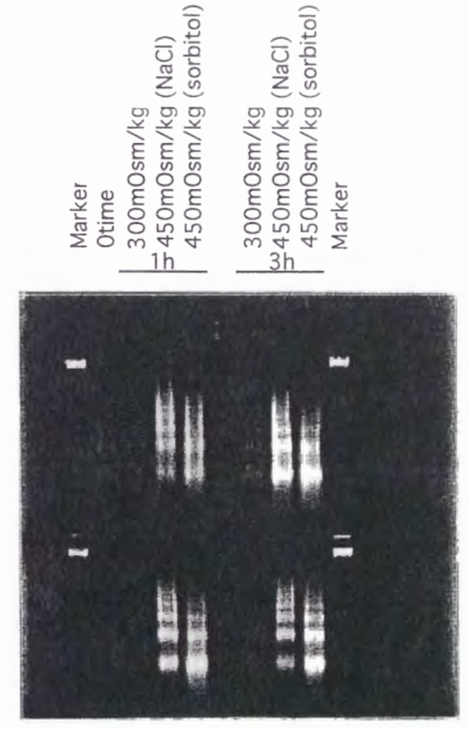


Fig. 1-7 Effect of Hyperosmolarity by Sorbitol Supplementation on Apoptosis.

DNA fragmentation was analyzed in the cultures exposed to hypertonic media with sodium chloride (NaCl) or sorbitol for 1 h, 3 h, 5 h, and 7 h. The culture before exposure is shown as 0 time.

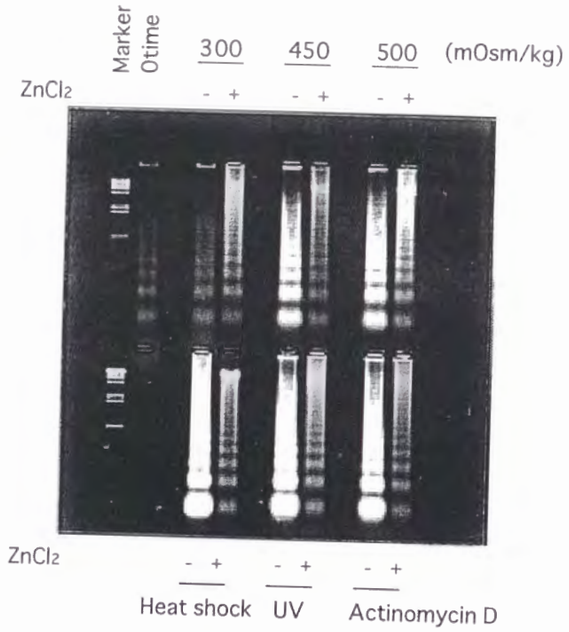


Fig. 1-8 Inhibition of DNA Fragmentation by Zn^{2+}.

Effect of Zn^{2+} was examined by DNA fragmentation assay. The samples were prepared from the cultures exposed to 300 (control), 450 and 500 mOsm/kg media, heat shock (40℃), UV irradiation (80 J/m^2), and actinomycin D (2 μg/ml) for 3 h in the absence or in the presence of 1 mM $ZnCl_2$. The culture before exposure is shown as 0 time.

Section 3
Suppression of Hypertonicity-induced Apoptosis by Acclimatization of EPC to Moderate Hypertonicity

Apoptosis is a physiological and pathological mode of cell death in an active and inherently controlled manner.[7, 9, 10] Apoptotic cell death is accompanied by nuclear condensation and DNA fragmentation, which, therefore, are defined as hallmarks of apoptosis.[7] By evaluating these features of apoptosis, the author has shown that a fish cell line EPC (*Epithelioma Papulosum Cyprini*)[2] undergoes cell death by apoptosis in response to hypertonic stress as well as other environmental stresses such as heat shock and UV irradiation (in Section 2). It has been reported that hypertonic stress can cause apoptosis also in mammalian cells.[37]

Previously, the author has reported that the extent of DNA fragmentation of EPC decreased from a significant level in a short period (3 h) of culture in hypertonic medium (supplemented with 2-fold inorganic salt constituents as much as usual, 470-480 mOsm/kg) to an undetectable level within a long period (6 days) (in Section 1). Furthermore, although growth inhibition and/or cell death was seen in EPC during the first 2 days in the hypertonic medium, the growth rate was recovered afterwards (in Section 1). These findings suggested that EPC undergoes apoptotic cell death with a short term exposure to the hypertonicity and then acclimates to the hypertonic environment during a long term culture. In contrast, hypotonic stress did not trigger cell death by apoptosis in EPC (in Section 2). Cell growth in a hypotonic medium (supplemented with inorganic salt constituents at a half concentration of the physiological medium, 200-210 mOsm/kg) was not significantly reduced in comparison with that in the physiological medium, 300 mOsm/kg).

In this study, the author first investigated whether EPC acclimates to hypertonic media supplemented with sodium chloride at various concentrations, evaluating cell growth and apoptotic cell death in each medium. Then, the author examined in detail DNA fragmentation with exposure to various osmotic media, employing the culture of EPC acclimatized to moderate hyper- or hypotonic medium.

Materials and Methods

Cell Growth in Different Osmotic Media

EPC was inoculated in a 35-mm plastic dish in MEM supplemented with 10% FBS at a density of 8×10^5 cells per dish and cultured for 15 h. The osmotic condition was changed by replacing the medium with each of the various osmotic media (Day 0 in Fig. 1-9). Further incubation was followed until measurement of the cell number, which was conducted with a haemocytometer according to a method described previously.[6] Three dishes were measured for each of the experimental media. The paired Student's *t*-test was done on the cell number per dish on Day 6 between 300 mOsm/kg and each of the other media.

Media and Phosphate Buffered Saline

A wide range of different osmotic media (150-600 mOsm/kg) supplemented with 10% fetal bovine serum (FBS, Biological Industries) and 2.5 mM HEPES (*4*-(*2*-hydroxyethyl)-*1*-piperazineethanesulfonic acid) were prepared as described in Section 2. Only the concentration of sodium chloride was different among the various osmotic media. Three phosphate-buffered salines (PBSs) were prepared by diluting 20-fold concentrated stock solution to the final osmotic pressures of 200, 300, 400 mOsm/kg. Osmotic pressure of all the media and PBSs was measured with Osmometer OM-801 (Vogel).

Acclimatization of Cells toHyper- or Hypotonic Media

A culture of EPC in 300 mOsm/kg medium attaining confluence in a 75-cm^2 culture flask (Nunc) was trypsinized, split equally to three 75-cm^2 culture flasks, and cultured for 5 days. The medium of each flask was replaced with either 200, 300, or 400 mOsm/kg medium and incubated for 4 days. Then all three cultures (200 mOsm/kg culture, 300 mOsm/kg culture , and 400 mOsm/kg culture) were subcultured at a split ratio of 1 : 2 by trypsinization using 0.2% trypsin in 200, 300, or 400 mOsm/kg PBS for each of the corresponding osmotic cultures. These cultures were used for the experiments after further 4 day incubation. All the cultures were done at 30℃ in air.

Exposure of EPC to Osmotic Stresses

EPC which was acclimatized to either 200, 300, or 400 mOsm/kg medium were inoculated in a 35-mm plastic dish at a density of 1×10^6 cells per dish in 200, 300, or 400 mOsm/kg medium, respectively. After 3 day culture, the cells in each of the three cultures were exposed to hypo- or hyperosmotic stress by replacing the medium with either 150, 200, 300, 400, 450, 500, or 600 mOsm/kg medium, and were incubated for 3 h.

In the same series of the experiment, before the exposure to the stress, the cell number in each of the cultures was measured with a haemocytometer by employing separate dishes of the same cultures used for DNA fragmentation assay. The cell number were shown by calculating the means from three independent dishes (Table 1).

Results and Discussion

Cell Growth of EPC under Various Osmotic Pressures

Effects of sodium chloride concentration in culture media on the cell growth of EPC were examined during the culture period of 6 days (Fig. 1-9). Because EPC is a cell line which has been subcultured in Eagle's minimum essential medium (MEM, 300 mOsm/kg) supplemented with 10% FBS for a long period,[2] 300 mOsm/kg medium seemed to be favorable for the growth of EPC. But unexpectedly, as a result, no significant difference was seen between the cell growth in 300 and 400 mOsm/kg media. In 200 mOsm/kg medium EPC cells showed an equal or higher growth rate as compared with those in 300 mOsm/kg medium. The cell growth was arrested until day 1 in 150 mOsm/kg medium. In 450 and 500 mOsm/kg, some portion of the cell population died by day 1. However, the remaining population seems to have grown, though the average growth rates were significantly lower than those in 300 mOsm/kg medium. Similar growth curves were observed for the cultures in both hyper- and hypotonic media compared with the cultures in the media at various concentrations of all inorganic salts (in Section 1).

DNA Fragmentation by Long Term Exposure to Various Osmotic Media

The previous study has shown that EPC cells underwent cell death by apoptosis and exhibits fragmented DNA in 400-600 mOsm/kg media particularly within 3 h (in Section 2). In order to know whether DNA fragmentation by apoptosis was detected during 6 day culture in various osmotic media, DNA fragmentation assay was done 1, 2, 4, and 6 days after

exposure to 150, 300, 400, and 450 mOsm/kg medium (Fig. 1-10). One fourteenth of the solubilized DNA from the whole culture was electrophoresed. Therefore, the extent of DNA fragmentation in this assay implicates an absolute amount of fragmented DNA in the whole culture because the cell number in the culture changed during these 6 days (Fig. 1-9). As a result, in 300 mOsm/kg, increasing amounts of fragmented DNA were observed, which seems to be parallel to the increasing cell population (Fig. 1-10). In contrast, although the cell population was growing, the amount of fragmented DNA in the whole culture of 400 mOsm/kg decreased gradually from a level of significant DNA fragmentation on day 1 to an undetectable level until day 6. In 450 mOsm/kg, the extent of DNA fragmentation was rather decreasing. Any significant DNA fragmentation was not seen in 150 mOsm/kg, while the fragmentation appeared on day 6, when the cell growth had been arrested. These observations suggest that EPC in the 400 mOsm/kg culture underwent some physiological changes during the culture period to acclimate to hypertonic environment. Almost complete elimination of fragmented DNA in the culture of 400 mOsm/kg might be due to an acceleration in phagocytosis of the apoptotic bodies by neighboring cells, which is generally found as one of the steps of apoptosis for self-clean up mechanisms.[21]

Acclimatization of EPC to Hyper- or Hypotonic Medium

The author prepared the culture of EPC acclimatized to moderate hyper- (400 mOsm/kg) or hypotonic (200 mOsm/kg) medium in order to know in detail the effects of hypertonic acclimatization of EPC on apoptotic cell death with exposure to severer hypertonic media. In this course of experiment, the author observed that at the passages the proportions of the the cell population which attached to the dish in moderate hyper- or hypotonic medium were smaller than that in the isotonic medium. Because of the decrease of attachment rates, there was a need to consider the size of the culture acclimatized to each of the media, although no significant difference in the growth rates of the EPC cultures in hypotonic (200 mOsm/kg), isotonic (300 mOsm/kg), and hypertonic (400 mOsm/kg) media during 4 day culture (Fig. 1-9). Different from the case when inoculated in 300 mOsm/kg medium, EPC exhibited decreased cell number per dish in 200 or 400 mOsm/kg medium after 3 day incubation when acclimatized to and

inoculated in 200 and 400 mOsm/kg, respectively (Table 1). Therefore, in the DNA fragmentation assays described below, the samples from the 200, 300, or 400 mOsm/kg-acclimatized cultures were adjusted to contain the same number of cells per lane on an agarose electrophoresis.

Effect of Osmotic Changes on DNA Fragmentation in the EPC Cultures Acclimatized to Different Osmotic Pressures

Each of the accllimatized cultures in 200, 300, or 400 mOsm/kg was exposed to 150-600 mOsm/kg media for 3 h by replacing the medium. In order to know the extent of apoptosis, DNA fragmentation was examined according to the method described in Materials and Methods. Because the cell numbers of the 200, 300, and 400 mOsm/kg-acclimatized cultures on the day examined were different (Table 1), the solubilized cell lysate containing fragmented DNA from the same number of the cells, 8.0×10^5, was electrophoresed on an agarose gel.

In the case of the 300 mOsm/kg-acclimatized culture, as the author reported previously (in Section 2), a slight DNA fragmentation was constantly seen in the culture before and after the exposure to the medium of the same osmotic pressure, 300 mOsm/kg (Fig. 1-11). In comparison with the extent of DNA fragmentation before the exposure, no significant increase in fragmentation was observed after the osmotic change to 150 or 200 mOsm/kg medium. In contrast, the exposure to hypertonic medium, 400-600 mOsm/kg, led to significant increase in the fragmentation in an osmotic-dependent manner. Our previous study indicated that the exposure to 600 mOsm/kg medium after 3 day pre-incubation in 300 mOsm/kg medium resulted in the decrease of DNA fragmentation probably because of cell death by necrosis (in Section 2). Similarly in this study, however, the exposure to 600 mOsm/kg medium induced cell death by necrosis as is demonstrated below.

Interestingly, DNA fragmentation in the case of the 400 mOsm/kg-acclimatized culture was unobservable with the exposure to 150-500 mOsm/kg media (Fig. 1-11). Significant DNA fragmentation was detected only in the culture exposed to 600 mOsm/kg, where the extent of the fragmentation was much smaller than that of the 300 mOsm/kg-acclimatized culture. These results suggest that the threshold of apoptosis against hypertonic stress was shifted to the higher osmotic pressure by the

acclimatization of the cells to the hypertonic medium of 400 mOsm/kg. However, hypotonic stress did not bring about cell death either by apoptosis or necrosis in spite of the hypertonic acclimatization, suggesting that the shift of the threshold of apoptosis by hypertonic acclimatization did not influence the tolerance of EPC against hypotonic stress.

The cultures which had been acclimatized to 200 mOsm/kg medium underwent a severer apoptotic event with the exposure to hypertonic media, 400-600 mOsm/kg (Fig. 1-11). The exposure to 300 mOsm/kg medium also resulted in DNA fragmentation, although at a very faint level. DNA fragmentation in the 200 mOsm/kg-acclimatized cultures exposed to 400, 450 and 500 mOsm/kg media were more remarkable than those in the 300 mOsm/kg-acclimatized cultures. In contrast to the 400 mOsm/kg-acclimatized culture, the threshold of apoptosis against hypertonic stress seemed to be shifted to the lower osmotic pressure by the acclimatization to the hypotonic medium of 200 mOsm/kg.

Acclimatization to different osmotic media affected the extent of DNA fragmentation by the same osmotic media. The acclimatization to moderate hypertonic medium of 400 mOsm/kg seemed to equip EPC to resist severer hypertonicity, while that to moderate hypotonic medium of 200 mOsm/kg seemed to enhance an apoptotic event with hypertonicity. For one possibility, as discussed above, the threshold to induce apoptosis against hypertonic stress is determined according to the absolute osmotic pressure and it could be shifted by acclimatization. However, for another possibility, this phenomenon could be due to difference in the relative changes of osmotic pressure. For instance, the exposure to 450 mOsm/kg caused different osmotic changes of 250, 150, and 50 mOsm/kg for the 200, 300, and 400 mOsm/kg-acclimatized cultures, respectively. According to the latter possibility, suppression of apoptosis in the 400 mOsm/kg-acclimatized culture with the exposure to 450 mOsm/kg medium might be due to the smaller relative change of osmotic pressure.

In order to clarify whether induction and suppression of apoptosis in the cultures acclimatized to the different osmotic media were caused by the relative changes in osmotic pressure, or by the absolute osmotic pressures, we attempted to exclude the difference in relative osmotic changes in a moment. Before the exposure to various osmotic media, all the cultures were preincubated for 1 h in 300 mOsm/kg medium. If the relative osmotic change

was an important factor to trigger apoptosis, all the cultures of the 200, 300, and 400 mOsm/kg-acclimatized cells would exhibit the same extent of DNA fragmentation because of the same relative osmotic change of 150 mOsm/kg. As a result, the extents of DNA fragmentation detected for all the 200, 300, and 400 mOsm/kg-acclimatized cultures were different, which gave the same pattern of the fragmentation as in Fig. 1-11 (Fig. 1-12). This suggests that acclimatization to hypertonic medium worked as a preparation to resist the stress of the absolute osmotic pressures of 400-600 mOsm/kg. In contrast, acclimatization to hypotonic medium reduced the tolerance against the absolute osmotic pressures of 400-600 mOsm/kg.

To evaluate the extent of cell death by necrosis, insoluble fractions of the cell lysate were examined by the electrophoresis (Fig. 1-12). This assay has been shown to be useful for estimating the decrease in genomic DNA in the culture which correlates with the extent of necrosis (in Section 2). Significant decrease in genomic DNA was observed in the 200 mOsm/kg-acclimatized culture with the exposure to 500 or 600 mOsm/kg, and in the 300 mOsm/kg-acclimatized culture with the exposure to 600 mOsm/kg. In these cases, a large portion of the culture is thought to have undergone cell death by necrosis in addition to apoptosis.

In this study, the author demonstrated for the first time that a fish cell line EPC acclimatizes to hypo- and hypertonic environments and shows different responses of apoptotic events to hypertonic stress. With the exposure to 500 mOsm/kg medium, significant extent of DNA fragmentation was observed in the 200 and 300 mOsm/kg-acclimatized cultures, while the 400 mOsm/kg-acclimatized culture gave no detectable amount of fragmented DNA. Significant DNA fragmentation was seen even in the 400 mOsm/kg-acclimatized culture with the exposure to 600 mOsm/kg. Because the exposure to 600 mOsm/kg led to complete elimination of genomic DNA, the whole population of the 200 mOsm/kg-acclimatized culture underwent cell death either by apoptosis or necrosis (Fig. 1-12). Acclimatization to the moderate hypo- or hypertonic medium seems to shift the threshold of the absolute osmotic pressure against both apoptotic and necrotic events in hypertonic environments.

Hypotonic stress did not induce apoptosis at a significant level in all the cultures of 200, 300, and 400 mOsm/kg-acclimatized cells. Although the threshold of apoptosis against hypertonic stress was shifted up to the higher

osmotic pressure by the acclimatization to 400 mOsm/kg medium, the 400 mOsm/kg-acclimatized culture was still tolerant to hypotonic environment. It has been reported that hypotonic medium was more preferable for EPC based on the cell growth (in Section 2).[8] His findings together with the previous reports suggest that EPC has an intrinsic viability in the hypotonic environment regardless of acclimatization.

Table 1 Cell Growth in Different Osmotic Medium.

Acclimatized to	200 mOsm/kg	300 mOsm/kg	400 mOsm/kg
Cell Number / Dish	3.55×10^6	6.44×10^6	3.73×10^6

The three different cultures acclimatized to 200, 300, or 400 mOsm/kg medium were inoculated at a density of 1×10^6 cells per dish in the corresponding osmotic medium and incubated for 3 days. The number of cells per dish was examined after 3 day incubation. Standard deviations are indicated as bars. The cell number in 200 or 400 mOsm/kg medium is statistically different from that in 300 mOsm/kg medium ($p<0.005$ for the paired Student's t-test).

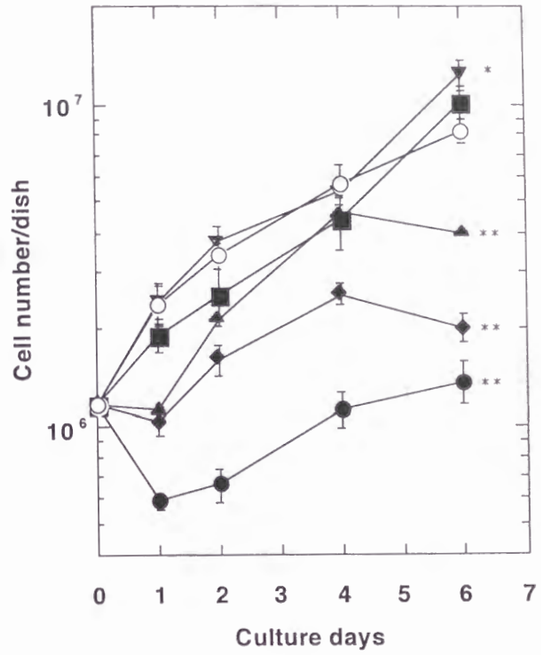


Fig. 1-9 Cell Growth in Different Osmotic Medium.
The number of cells per dish was examined on days 1, 2, 4, and 6 in each of 150 (▲), 200 (▼), 300 (○), 400 (■), 450 (◆), and 500 (●) mOsm/kg media. Standard deviations are indicated on all the points as bars, although some of them are too small to be shown. Significant differences determined on day 6 by the paired Student's t-test are shown as stars (*: $p<0.05$, **: $p<0.005$). The cell number in 200 or 400 mOsm/kg medium was not statistically different from that in 300 mOsm/kg medium until day 4 or day 6, respectively.

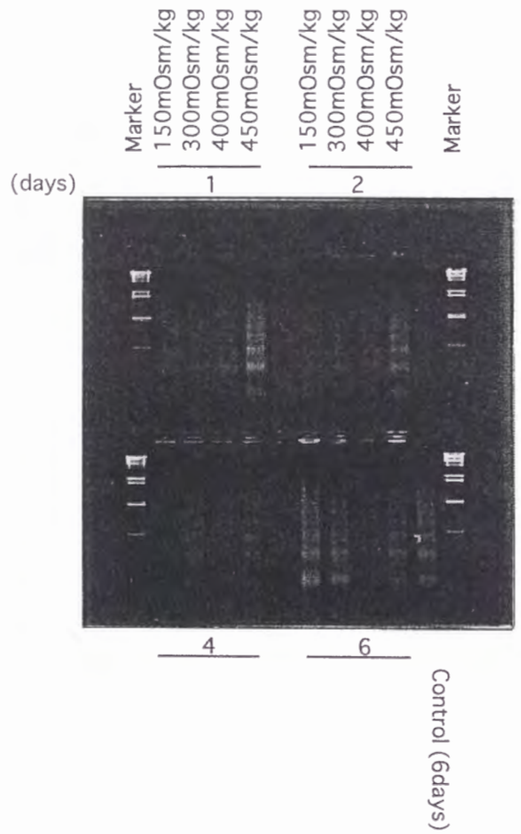


Fig. 1-10 DNA Fragmentation by Long Term Exposure to Various Osmotic Media.
DNA fragmentation assay was done using the same portion of each of the whole culture. The cultures incubated in 150, 300, 400, and 450 mOsm/kg media were analyzed 1, 2, 4, and 6 days after medium-replacement. The culture incubated for 6 days without any medium-replacement was shown as a control.

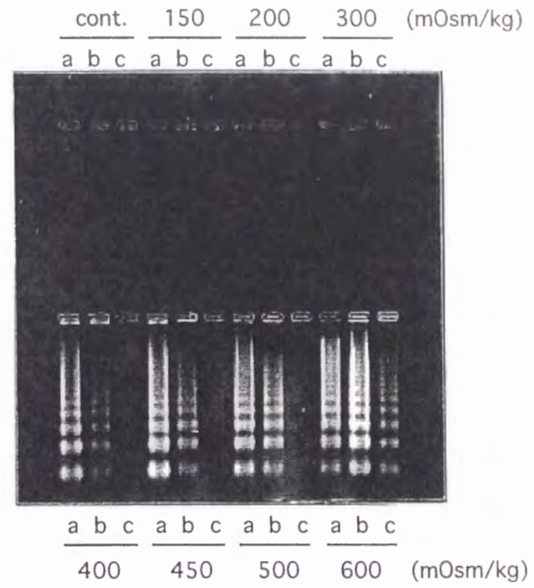


Fig. 1-11 Suppression of DNA Fragmentation by Acclimatization.
The three different cultures acclimatized to 200 (a), 300 (b), or 400 (c) mOsm/kg medium were inoculated at a density of 1×10^6 cells per dish in the same osmotic medium and incubated for 3 days. DNA fragmentation was examined at 3 h after the exposure to 150, 200, 300, 400, 450, 500, or 600 mOsm/kg medium. The cell lysate of 8×10^5 cells from each of the cultures was electrophoresed on an agarose gel. Each of the 200, 300, and 400 mOsm/kg-acclimatized cultures before the exposure was examined as a control.

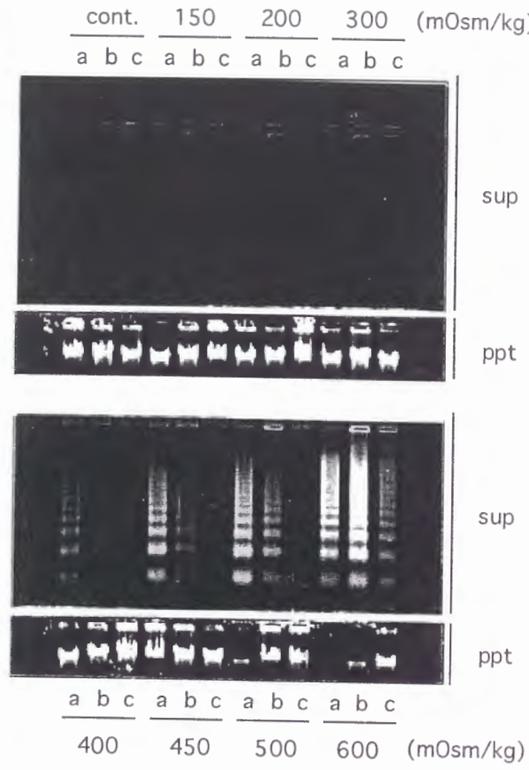


Fig. 1-12 Effects of Pre-incubation in 300 mOsm/kg Medium on the Extent of Apoptosis.

The conditions of the experiment were the same as given in the legend of Fig. 1-11 except for the addition of 1 h-preincubation of all the cultures in 300 mOsm/kg medium before the exposure. The soluble (sup) and the insoluble (ppt) fractions of the cell lysate were both electrophoresed.

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CHAPTER II

The JNK/SAPK MAP Kinase Pathway in Carp

Section 1
Structure and Expression of Carp JNK/SAPKs (cJNKa and cJNKb)

MAP kinase (mitogen-activated protein kinase) pathway, also referred to as the classical MAPK or the ERK pathway, consists of a protein kinase cascade linking extracellular signals, such as growth and differentiation stimuli, with transcription in the nucleus.[1] In response to the stimuli, MAP kinase is activated through its phosphorylation on Thr and Tyr residues at the Thr-Glu-Tyr dual phosphorylation motif catalyzed by MAPKK, referred to as MEK, whose activation requires serine phosphorylation catalyzed by MAPKKK, such as Raf.[2] MAP kinase which becomes activated by this chain of phosphorylation relayed by the upstream protein kinases is translocated from the cytoplasm to the nucleus and phosphorylates nuclear proteins including transcription factors. This nuclear translocation of activated MAP kinase appears to be crucial for regulatory features of MAP kinase cascade on gene expression.[3-5]

JNKs, JNK1 and JNK2 (p46 and p54), was initially identified as protein kinases which were remarkably activated by UV irradiation.[6] Afterwards, the cDNAs of JNKs, also termed SAPKs, have been identified in man and rat.[7-10] DNA sequence analysis of these cDNAs revealed that JNK/SAPK could be classified into the MAP kinase super family and shared some similarity with yeast HOG1, which had been identified as a MAP kinase essential for growth in a hypertonic environment.[11] Complementation of HOG1-mutation by introducing JNK1 cDNA in yeast supported the functional similarity between JNK1 and HOG1.[12] Indeed, JNK1 was activated by hypertonic stress in mammalian cells.[12, 13] However, a significant difference is seen between JNK1 and HOG1 in the dual-phosphorylation motif, Thr-Pro-Tyr and Thr-Gly-Tyr, respectively. JNK/SAPK homolog with the Thr-Pro-Tyr motif has not been identified in lower animals or yeast, while mammalian p38s[14, 15] and frog Mpk2[16] share with HOG1 the same motif, Thr-Gly-Tyr. These findings are of interest in relation to the molecular evolution of the JNK/SAPK subgroup of the MAP kinase

superfamily.

Here the author reports the molecular cloning of two stress-activated protein kinase (cJNKa and cJNKb) cDNAs from carp. Tissue differences in expression of cJNKa and cJNKb were also examined.

Materials and Methods

Preparation of RNA

Total RNA from either a carp epithelial cell line, EPC,[17] or various tissues of a female carp (*Cyprinus carpio* L.) was isolated by ultracentrifugation in 5.7 M cesium chloride after homogenization in 4 M guanidinium thiocyanate.[18] Poly $(A)^+$ RNA was enriched by chromatography either on oligo (dT) cellulose (Collaborative Biomedical Products) or on Oligotex-dT30 (Takara).

RT-PCR

Two pairs of oligonucleotide primers were prepared: J-1 pair (sense: 5'-AANCGATAYCARAAYYT-3', and antisense: 5'-NAWNACNAGCATYTT-3') and J-2 pair (sense: 5'-GTNGCNATHAARAARCT-3', and antisense: 5'-CAAYTGRTCDATAT-3'); (abbreviations according to the IUPAC code). These primers were designed from the well-conserved sequence of the MAP kinase family, human JNK1,[7] mouse p38,[14] mouse p42,[19] and yeast HOG1.[11] The J-1 pair corresponds to the amino acid sequences (K, E, N, P)RY(Q, N, T)(N, D)L (residues 24-29 of human JNK1) and KML(V, T)(I, L, F) (residues 300-304). The J-2 pair corresponds to the amino acid sequences VA(I, V)KK(L, I) (residues 52-57 of human JNK1) and (Y, H)(I, V, L)(D, H)Q(L, W, F) (residues 229-233).

Single-stranded cDNA was synthesized from 1 μg of total RNA with 200 units of MMLV-RT (Gibco-BRL) in 20 μl of a reaction mixture containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, 0.1 mg/ml BSA, 1 mM each dNTP, 5 μM random hexamer, and 20 units of ribonuclease inhibitor RNasin (Toyobo). A quarter of the resultant cDNA was used as a template in the first PCR, which was carried out in a 25 μl solution containing 8 units of ΔTth DNA polymerase (Toyobo), 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, 0.1 mg/ml BSA, 0.2 mM each dNTP, and 0.2 μM each primer of the J-1 pair. The second PCR was performed using 1 μl of the first PCR reaction mixture in 50 μl containing 2.5 units of ΔTth DNA polymerase, 25 μM each primer of the J-2 pair, and 0.2 mM each dNTP. The conditions of both PCRs were 30 s at 95℃, 1 min at 56℃, and 2 min at 72℃ for 25 cycles (Astec, Program Temp. Control System PC-700).

cDNA Cloning

Synthesis of double-stranded cDNA was accomplished with a ZAP-cDNA Synthesis Kit (Stratagene) using poly $(A)^+$ RNA prepared from the ovary by chromatography on oligo (dT) cellulose. cDNAs were size-fractionated with a CHROMA SPIN-100 column (CLONTECH) and adapter-ligated prior to generation of oligo (dT) primed library in Uni-ZAP/*Eco*RI/*Xho*I/CIAP (Stratagene).

The cDNA library was screened with a 549 bp fragment obtained by PCR as a probe. Plaque hybridization was done at 42℃ in a solution containing 5×SSC (1×SSC=150 mM sodium chloride, 15 mM sodium citrate, pH 7.0), 0.1% SDS, 50% formamide, 100 μg/ml denatured salmon sperm DNA, 0.6% Ficoll 400, 0.6% polyvinylpyrrolidone 100, 0.6% BSA, and ^{32}P-labeled probe prepared by use of the Megaprime DNA labelling system (Amersham International plc). After hybridization, the filters were washed four times with 2×SSC at room temperature and three times with 2×SSC, 0.5% SDS at 65℃ prior to autoradiography. Plasmids (pBluescript SK) containing the cDNA insert were obtained by *in vivo* excision from positive single plaques according to the manufacturer's instruction (Stratagene). After subcloning into pBluescript II KS (Stratagene), DNA fragments were sequenced by a PCR procedure employing fluorescent dideoxynucletides and a model 373A automated sequencer (Applied Biosystems Inc.). The sequences reported in this paper were determined on both strands.

Southern and Northern Blot Analyses

A genomic Southern experiment was performed by the standard procedure[18] using 10 μg of carp genomic DNA digested with restriction enzymes (Toyobo). Northern analysis was carried out using poly $(A)^+$ RNA prepared by Oligotex-dT30 (Takara). In both analyses, the probes for hybridization were prepared from the 549 bp cDNA fragment, or 3' untranslated region of the two distinct JNK cDNAs. Hybridization was done under the same conditions described above.

Detection of Two Distinct JNK mRNAs

RT-PCR assay was performed with primers having the following sequences: The sense primer (J5') specific to nucleotides 212-231 of the ORF of both cJNKa and cJNKb cDNAs is 5'-ACAGAGAGCTGGTGCTCATG-3'. The antisense primers specific to nucleotides 660-679 of the ORF of cJNKa and cJNKb cDNAs are 5′-CAGGAAAAAGGATTTTGTGA-3′ (Ja) and 5′-CTGGAAACAACACACTACCT-3′ (Jb), respectively (Fig. 2-2). Fifty ng of poly $(A)^+$ RNA purified by Oligotex-dT30 (Takara) was transcribed into cDNA with 5 units of AMV-RT (Takara) in 20 μl of a reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 1 mM each dNTP, 2.5 μM random nonamer, and 5 units of ribonuclease inhibitor (Takara). One-tenth of the resultant cDNA was used as a template for PCR, which was carried out in a 10 μl solution containing 0.5 units of recombinant Taq DNA polymerase (Takara), 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, 0.1 mg/ml BSA, 0.2 mM each dNTP, 0.2 μM each primer of J5' and either Ja or Jb. For an internal control, β-actin mRNA was amplified with a pair of oligonucleotides (sense: 5'-TGCCATCCAGGCTGTGCTG-3', and antisense: 5'-CCATCTCCTGCTCGAAGT-3') as primers. The conditions of PCRs were 30 s at 94℃, 30 s at 60℃, and 1 min 30 s at 72℃ for 30 cycles (Astec, Program Temp. Control System PC-700). Amplified PCR fragments were stained with ethidium bromide after electrophoresis on a 1% agarose gel.

Results and Discussion

cDNA Cloning of cJNKa and cJNKb

The cDNA amplification by RT-PCR using a primer pair (J-1 set) in the first PCR, followed by the second PCR employing the other primer pair, J-2 set, yielded an expected 549 bp band corresponding in size to a potential JNK. Indeed, a homology search with the GenBank database revealed that the cDNA fragment was homologous to the inner part of human JNK1 cDNA.[7]

The author first examined the tissue distribution of JNK mRNA in carp by Northern blot analysis with the PCR fragment as a probe. As a result, expression of 4.5 kb mRNA was detected in the brain and ovary (Fig.

2-1). The ovary showed a stronger signal of the 4.5 kb transcript than the brain, and in addition a faint signal of a smaller transcript whose length was approximately 3 kb. Therefore, in order to isolate the full length cDNA for carp JNK, The author constructed a cDNA library using ovary RNA. The expression of JNK mRNA was undetectable in the gill, heart, spleen, liver, kidney, muscle, gut, and gall bladder by Northern blot analysis.

The cDNA library was screened with the PCR fragment as a probe. Screening of approximately 3×10^5 plaques yielded 11 positive clones. Restriction mapping and sequence analysis of both the 5'- and 3'-ends of the clones showed the presence of two distinct classes, cJNKa and cJNKb (Fig. 2-2). The amino acid sequence predicted by the nucleotide sequence of the largest clone from one class (cJNKa) contained 427 residues with a predicted mass of 48.6 kDa (DDBJ accession number D83273, Fig. 2-3). The in-frame stop codons in the 5' and 3' regions of the cDNA indicated that this clone contains the entire carp JNK coding region. cJNKb cDNA also encoded the same length of ORF, while the nucleotide sequence was different from that of cJNKa: The homology in nucleotide sequence of the ORF is 93.6% (DDBJ accession number AB001744).

Multiple alignments of amino acid sequences of cJNKs (cJNKa and cJNKb) with those of other animals are shown in Fig. 2-4. When the sequence of cJNKa is compared with that of cJNKb, 95.8% of the residues are identical; among 427 residues in the deduced sequence, only 18 residues are different. MAP kinases are known to be activated through dual phosphorylation of adjacent threonine (Thr) and tyrosine (Tyr) residues.[20] These putative phosphorylation sites are Thr-183 and Tyr-185 in both of the carp JNKs. These sites of carp JNKs possess the Thr-Pro-Tyr tripeptide motif, which is conserved in the JNK subgroup of the MAP kinase family.[1, 2, 21] Comparison with protein sequences in the SWISS-PROT database by FASTA indicated that cJNKa is 87.4%, 77.3%, 76.8%, 83.6%, and 86.1%, and cJNKb is 87.1%, 79.4%, 78.2%, 83.1%, and 86.1%, identical in primary structure to human JNK1[7] and JNK2,[8, 9] and rat SAPKα, β and γ,[10] respectively. The carboxy-terminal region of carp JNKs is 43 and 16 residues longer than those of human JNK1 and rat SAPK γ, respectively, although the homologies are relatively high. When compared in terms of the sequences and lengths at the carboxy-terminal region, carp JNKs are similar to human JNK2 and rat SAPKβ (Fig. 2-4). These similarities suggest that cJNKa and

cJNKb belong to the JNK/SAPK subgroup of the MAP kinase superfamily. It should be noted that carp JNK isoforms have the same length of deduced amino acid sequence, although mammalian JNK/SAPK isoforms differ in length as shown in Fig. 2-4.

Difference in Tissue Distribution between cJNKa and cJNKb

The cDNA fragment isolated by RT-PCR possibly probed both cJNKa and cJNKb mRNAs in the Northern blot analysis of various tissues (Fig. 2-1), because of the sequence similarity in the inner part of the ORFs of the cloned cJNKa and cJNKb cDNA. Actually, multiple bands were observed by Southern blot analysis of carp genomic DNA using the fragment as a probe, indicating that the probe hybridized to both cJNKa and cJNKb genes (data not shown). In order to clarify whether there was a difference in length between cJNKa and cJNKb mRNAs, by using the 3'-untranslated regions of cJNKa and cJNKb cDNAs (Fig. 2-2) as a probe, The author carried out Northern blot analysis with poly (A) rich RNA of the ovary, where two lengths of the transcript, 3 kb or 4.5 kb, were detected (Fig. 2-1). The specificity of these probes was confirmed by a genomic Southern blot analysis to distinguish cJNKa and cJNKb mRNAs (Fig. 2-5). Figure 2-6 shows that both of cJNKa and cJNKb probes strongly recognized the 4.5 kb mRNA, suggesting that the transcripts of cJNKa and cJNKb do not vary in length. The faint signal of the smaller mRNA of 3 kb (Fig. 2-1) possibly implies the existence of a related gene, unidentified at this time.

In order to investigate in detail the tissue distribution of cJNKa and cJNKb mRNAs, The author carried out RT-PCR assay, in which a higher sensitivity and specificity of detection could be expected. Two different antisense primers in combination with a common sense primer were employed to distinguish cJNKa and cJNKb mRNAs. Figure 2-7 shows that mRNA expression of cJNKa and cJNKb exhibited different patterns of tissue distribution. cJNKb mRNA was expressed in every tissue examined, being most abundant in the ovary. In contrast, the expression of cJNKa mRNA was detected only in the brain and ovary. cJNKa mRNA was more abundant in the brain than cJNKb mRNA. These findings suggest that cJNKa and cJNKb are expressed under different transcriptional regulations among carp tissues.

In mammals, osmotic stress, heat shock, UV irradiation, IL-1, TNF,

LPS and other stress factors were reported as candidate initiators of stress-responsive signal transduction through JNK/SAPK[2, 7-10, 12, 13, 21]. Since carp JNKs are highly conserved in structure compared with other animals, they may transduce environmental stress signals to the nucleus. Indeed, they were activated by UV irradiation and hyperosmotic stress (in Section 2). On the other hand, the existence of large amounts of cJNKa and cJNKb mRNAs in the ovary suggests a role of JNK mRNA or protein in embryos as a maternal factor. His findings in this study raise the possibility that a female carp equips eggs in the ovary with JNKs to aid their survival when exposed to various environmental stresses during ectogenetic early development. Since the fish embryos do not possess highly-organized endocrinological systems, such as a neurohormonal regulation, cellular responses to environmental stresses seem to be highly dependent on the JNK/SAPK stress-activated kinase pathway. In studies on the function of JNK at an early developmental stage, fish should be a good model for *in vivo* studies because external fertilization and ectogenetic early development, in addition to the transparency, make it possible to observe directly the inside of the embryos.[22, 23] This would be a great advantage in the application of fish as a vertebrate model, whereas there are difficulties in investigating the physiological functions of JNK/SAPK in mammalian embryos.

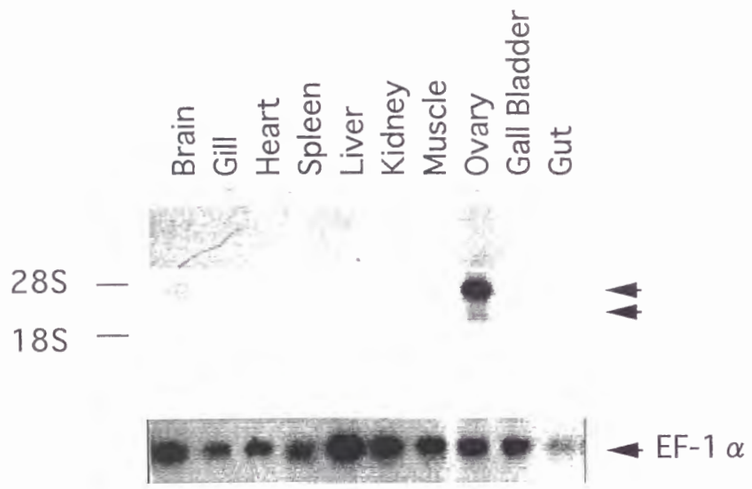

Fig. 2-1. Tissue Distribution of Carp JNK mRNA.

Poly (A)$^+$ RNA samples were prepared from the brain, gill, heart, spleen, liver, kidney, muscle, ovary, gall bladder, and gut. Each RNA sample (2 μg) was separated by 1% agarose-formaldehyde gel electrophoresis, transferred onto a nylon membrane and fixed. The JNK cDNA fragment derived by *RT-PCR* cloning was used as a probe. After hybridization, the membrane was washed at 65℃ in 2×SSC containing 0.5% SDS. The blot was reprobed with medaka elongation factor-1α cDNA as an internal control. The positions of 28S and 18S rRNAs are indicated on the left by arrowheads.

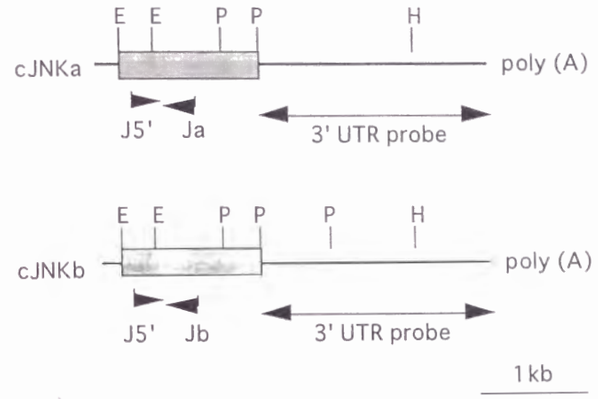

Fig.2-2. Restriction Map of the cDNA Clones of cJNKa and cJNKb.

Filled boxes indicate the ORFs. Restriction endonuclease cleavage sites are indicated as follows: H, *Hin*dIII; P, *Pst*I; E, *Eco*RI. Arrows below the ORFs show the position of primers, Ja, Jb, and J3', which were used in RT-PCR assay (Fig. 6). The 3'-untranslated region (UTR) used as a probe for Southern and Northern blot analyses is illustrated by a line with double arrowheads.

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-208                                                    GAAGAAAGCGCTTTCAATACAGTGAGGT   -181
-180 CATGTTATCGAGACAAGCAGCAGAATAAGCGCTATTATTAATCGATAGAGTCTTCGCCACATCGTTTCTGAAGAGTCCTGATATTTACGT   -91
 -90 GGCATTTAGGATAGCGGGAGTGTTTATATTTCAGTCGTCGCAGGTCTGTAAAAGTCTGCCTTGTTCGGATGCTCACACATCGGCTTCACC    -1

   1 ATGAACAAAAATAAGCGAGAAAAAGAATTCTACAGTGTCGATGTGGGTGATTCAACATTCACAGTGTTGAAGCGGTATCAGAATCTTAGA    90
   1 M  N  K  N  K  R  E  K  E  F  Y  S  V  D  V  G  D  S  T  F  T  V  L  K  R  Y  Q  N  L  R    30

  91 CCCATTGGCTCGGGTGCTCAGGGAATAGTCTGCTCAGCATATGACCACAACCTTGAGCGAAATGTGGCTATAAAGAAACTCAGCCGGCCT   180
  31 P  I  G  S  G  A  Q  G  I  V  C  S  A  Y  D  H  N  L  E  R  N  V  A  I  K  K  L  S  R  P    60

 181 TTTCAGAATCAGACTCATGCCAAACGCGCATACAGAGAGCTGGTGCTCATGAAGTATGTCAACCATAAAAACATAATATGTCTGTTAAAT   270
  61 F  Q  N  Q  T  H  A  K  R  A  Y  R  E  L  V  L  M  K  Y  V  N  H  K  N  I  I  C  L  L  N    90

 271 GTTTTTACGTCACAAAAAACATTAGACGAATTCCAAGACGTTTACCTAGTAATGGAGCTGATGGATGCCAACCTCTGCCAGGTCATTCAG   360
  91 V  F  T  S  Q  K  T  L  D  E  F  Q  D  V  Y  L  V  M  E  L  M  D  A  N  L  C  Q  V  I  Q   120

 361 ATGGAGCTGGATCACGAGCGGCTGTCCTATCTGCTTTACCAGATGCTGTGCGGAATAAAACACCTCCACTCGGCGGGGATCATCCACAGG   450
 121 M  E  L  D  H  E  R  L  S  Y  L  L  Y  Q  M  L  C  G  I  K  H  L  H  S  A  G  I  I  H  R   150

 451 GATCTGAAACCCAGTAACATTGTGGTAAAGTCAGACTGTACGCTGAAGATCCTGGATTTCGGTCTGGCCAGGACAGCAGCTACAGGTCTG   540
 151 D  L  K  P  S  N  I  V  V  K  S  D  C  T  L  K  I  L  D  F  G  L  A  R  T  A  A  T  G  L   180

 541 CTGATGACACCATATGTGGTGACACGTTACTACAGGGCCCCTGAAGTCATCCTGGGAATGGGATATCAAGCCAATGTGGACATTTGGTCT   630
 181 L  M  T  P  Y  V  V  T  R  Y  Y  R  A  P  E  V  I  L  G  M  G  Y  Q  A  N  V  D  I  W  S   210

 631 GTGGGCTGCATTTTGGCAGAAATGGTCCGTCACAAAATCCTTTTTCCTGGGAGGGACTATATTGATCAGTGGAACAAAGTCATAGAGCAG   720
 211 V  G  C  I  L  A  E  M  V  R  H  K  I  L  F  P  G  R  D  Y  I  D  Q  W  N  K  V  I  E  Q   240

 721 CTGGGAACACCAACGCAGGAGTTCATGATGAAACTCAACCAGTCTGTGCGAACCTATGTGGAGAACAGGCCGCGATACACTGGATACAGC   810
 241 L  G  T  P  T  Q  E  F  M  M  K  L  N  Q  S  V  R  T  Y  V  E  N  R  P  R  Y  T  G  Y  S   270

 811 TTTGAGAAGCTCTTTCCCGATGTCCTGTTCCCTGCTGATTCAGAACACAACAAACTGAAAGCAAGCCAGGCGCGGGACCTGCTGTCTAAA   900
 271 F  E  K  L  F  P  D  V  L  F  P  A  D  S  E  H  N  K  L  K  A  S  Q  A  R  D  L  L  S  K   300

 901 ATGCTGGTGATAGATGCATCCAAGCGAATCTCTGTGGAAGAAGCTCTGCAGCACCCGTACATTAACGTGTGGTACGACCCAGCTGAAGTG   990
 301 M  L  V  I  D  A  S  K  R  I  S  V  E  E  A  L  Q  H  P  Y  I  N  V  W  Y  D  P  A  E  V   330

 991 GAAGCGCCACCTCCTGTAATCACAGACAAACAGCTCGATGAGAGGGAACACACAGTGGAGGAGTGGAAAGAGCTGATCTATAAAGAAGTT  1080
 331 E  A  P  P  P  V  I  T  D  K  Q  L  D  E  R  E  H  T  V  E  E  W  K  E  L  I  Y  K  E  V   360

1081 CTGGATTGGGAAGAACGGATGAAAAACGGTGCCATTCGAGGTCAGCCTTCTCCTCTAGGTGCAGCAGTGATCAACGGCTCACCCCAGCCC  1170
 361 L  D  W  E  E  R  M  K  N  G  A  I  R  G  Q  P  S  P  L  G  A  A  V  I  N  G  S  P  Q  P   390

1171 TCCTCCTCTTCCTCCATCAATGACGTCTCCTCCATGTCCACGGAGCCCACCGTGGCCTCAGACACAGACAGCAGCCTTGAGGCCTCCGCA  1260
 391 S  S  S  S  S  I  N  D  V  S  S  M  S  T  E  P  T  V  A  S  D  T  D  S  S  L  E  A  S  A   420

1261 GGACCCCTGAGCTGCTGCAGATGACTATCACCTTCCCCTGGAGTTTCTCTGTGTGTGGCATGGTATCAGATTTAGGCCAGTCTAATACTT  1350
 421 G  P  L  S  C  C  R  *                                                                      428

1351 CATTTCGGGTCAGGTGTGGCTTTTTTGAGTTCCGCTCTAGATGCTGTAAATGACTACACAGTTTTATCTTTTTAGCAGCAGCCCCAATGA  1440
1441 TAGTGAACTCCTTAACAAATCTCTTCTTTTATAAGCTACGTTTAGGGTAATTTGTCAAGTTGTCGTAGAGTGATTTTGGAAGGTAATCTG  1530
1531 TAAAGATTGAATTGTGAATATATTTTCTCAAATTATTTTTTTCCTGGTCAATGTATAATTTTCTTGGTCACTTGGGATATGAAAATCTTT  1620
1621 AGAAACAAGTCCATATTAGTATTTTTAAATGGATATAACAATATTGCAAATTAATATCAGCATAGTTATTTAAGTTCATTTTGGCATAAC  1710
1711 CTGCCTGAGGTGGCTTTTTCTGGGTCATATTTTCCGTCATTGTGCGCAGCGTATTTTATTTAAATTTTGTACAGTTTCATTTAGATATAA  1800
1801 TAACTTACAACATCAAAAAAATATATTTTTTATGTACATTGATTTATTTTACCAGGATGTTCTTTTTTTCATGAATAGTTTTGCTGCTGG  1890
1891 TTTAATTTCCTGGTTGAATGTGAAATGTGACGTGCTTGTGCGTATGTCTCTGGTGTTCTAGTTTTTGTTCTAGTCCTAGTTACCTAGGAT  1980
1981 TTCAGCACCTTTTTTATTTTTTTATAATTAAAAAATTGTTTTACCTCTTCAAAATCTGCATATAGAACAAGCATTGTGCTTCATTATTGT  2070
2071 ATCATTGCATTTTGCATTTTTTTTCTCAAATGTTTTTTCCAACCCTTAATGAGGTTTGGTTTTGTGACATATGAGTCCGAAATTTCATGA  2160
2161 GCTGGTAGAACAAATGTTAATAATTTACAATCATTTTATTATTATTATTTGGAACACCAATAGAGTCTACACTGGTTCATTTTTAGCATA  2250
2251 TTCCATTAGAGTTTTAAGCTACTTATTTATTTTTATACAACAGTGAATGAACCAGATTTGAAAATAATTTATGTCTAGATCGGTTTTACT  2340
2341 TTGTTTTTTTTTCCATCATTTTGGAAGGTCTTTATTGGCATGTGATGTCCTGTAAATGAATGGGTGCTTTTTAACTTGTGTCATTAAATA  2430
2431 CTGTGGAGCGCCGCACATTATTTGATAAAACGTTTAAGAAAAATCAGGGAAGTGGGTTTCTTTTACATTTGCTTCTGAATGTGAAGGTGA  2520
2521 TAGAGAATGGCGAATCGGCCTGAAGTTGAGGAGTTTAAAGAGGGAATCTGAATGTTGTTGACATGGGGTTCAGCCCATTGGATATATTTA  2610
2611 CCTGTGCACAGGGTAAAAAACTCAATATTTTGCTATTACTTTATATTATTGCTGTTTTGTTTTAAGTGCTCTTAACAGTGGCAATGATAT  2700
2701 TAAACCCTACATACTTGCCTGGGAAGCCTGAGTCTGCTGTACCTGCAAAGAGCAAAAGCTTCTCAACATGTGATATGTTCTGGACATGAA  2790
2791 ACACAAAGCCGTTTGAAGGTCGAGTCTGAACTACCTCACACTGATACCATACTGCTCACATTTTAAAAGTATTATTGTAACCTTTATTTC  2880
2881 ATTTTATTTATTTATTCGGAAAAATAGGAGTGTTCAACTGTCTGTATAGTTTGAATCTATGAGAAAGTACTGTATGTATGTGTATACAAT  2970
2971 TCAGCGAGTATCTTTGTTTATTCTAATCTAGACTGTTTTTTTGTTTTCTTCAGCTTATGTGCAGTAACTCAAAGCTGAACAATGAACCAT  3060
3061 GTTATTTCAGACTCGACATCTATATGAAAATGTTTGGAAGGATTCTGTCCATGTTTTGCTGTTTCGAGAAAAAGAAATGAGAATTATTAAA  3150
3151 AAAAGAAGTGGAAAAGTTTCTCATCCCCAATCTCAACGATGGGCGGTATCTTGCATGTCCTGCTTAGCATTTGATTTTAAGTCCAGAACA  3240
3241 CTGTACAAGAACTATAAGATCATGGTGCTTTGTTTATTAGACTGCACAAGCTGATGCAGTTGTTTTATATTAGGGTTAGCAGATTACCAT  3330
3331 CTCATTAGAATGCACTGAAATTCAATTGTGTTACCATTATAGCAACTGGAAAAATGAAAATAAACAATTGGAAAATGAAAAAAAAAAAAA  3420
3421 AAAAA                                                                                       3425
```

Fig. 2-3 Nucleotide and Deduced Amino Acid Sequences of cJNKa.

The open reading frame (427 amino acids) is preceded by four in-frame stop codons, indicated by underlining. The putative dual-phosphorylation site is double-underlined. The predicted stop codon is denoted by an asterisk.


```
JNKa    carp      1 MNKNKREKEF YSVDVGDSTF TVLKRYQNLR PIGSGAQGIV CSAYDHNLER NVAIKKLSRP
JNKb    carp      1 .......... .......... .......... .......... .......... ..........
JNK1    human     1 .SRS..DNN. ...EI..... .........K .......... .A...AI... ..........
JNK2    human     1 .SRS.CDSQ. ...Q.A.... .......Q.K .......... .A.F.TV.GI S..V......
SAPKβ   rat       1 .S.S....Q. ...E...... .........K .......... .A...AV.D. ..........
SAPKγ   rat       1 .SRS..DNN. ...EIA.... .........K .......... .A...AI... ..........
HOG1    yeast     1 .T---TNE.. IRTQIFGTV. EITN..ND.N .V.M..F.L. ...T.TLTSQ P.....IMK.

JNKa    carp     61 FQNQTHAKRA YRELVLMKYV NHKNIICLLN VFTSQKTLDE FQDVYLVMEL MDANLCQVIQ
JNKb    carp     61 .......... ........C. ......G... ...P....E. .......... ..........
JNK1    human    61 .......... ........C. ......G... ...P..S.E. .....I.... ..........
JNK2    human    61 .......... ......L.C. ......S... ...P....E. .......... .........H
SAPKβ   rat      61 .......... ........C. ......S... ...P....E. .......... ..........
SAPKγ   rat      61 .......... ........C. ......G... ...P..S.E. .....I.... ..........
HOG1    yeast    58 .STAVL...T ....K.LKHL R.E.L...QD I.-----.SP LE.I.F.T.. QGTD.HRLL.

JNKa    carp    121 ME-LDHERLS YLLYQMLCGI KHLHSAGIIH RDLKPSNIVV KSDCTLKILD FGLARTAATG
JNKb    carp    121 ..-....... .........T .......... .......... .......... ..........
JNK1    human   121 ..-.....M. .......... .......... .......... .......... .......G.S
JNK2    human   121 ..-.....M. .......... .......... .......... .......... .......C.N
SAPKβ   rat     121 ..-.....M. .......SA. .......... .......... .......... .......G.S
SAPKγ   rat     121 ..-.....M. .......... .......... .......... .......... .......G.S
HOG1    yeast   113 TRP.EKQFVQ .F...I.R.L .YV....V.. .........LI NEN.D...C. .....IQDPQ

JNKa    carp    180 LLMTPYVVTR YYRAPEVILG -MGYQANVDI WSVGCILAEM VRHKILFPGR DYIDQWNKVI
JNKb    carp    180 .......... .......... -........V ......M... ..GSV....S .H........
JNK1    human   180 FM........ .......... -...KE...L ......MG.. .C........ ..........
JNK2    human   180 FM........ .......... -...KE.... ......MG.L .KGCVI.Q.T .H........
SAPKβ   rat     180 FM........ .......... -...KE.... ......MG.. .......... ..........
SAPKγ   rat     180 FM........ .......... -...KE...L ......MG.. .CL....... ..........
HOG1    yeast   173 --..G..S.. ......IM.T WQK.DVE... ..A...F... IEG.P....K .HVH.FSIIT

JNKa    carp    239 EQLGTPTQ-- EFMMKLNQSV RTYVENRPRY TGYSFEKLFP DVLFPADSEH NKLKASQARD
JNKb    carp    239 ......S.-- .......... .......... .......... .......... ..........
JNK1    human   239 ......CP-- ...K..QPT. ........K. A......... .......... ..........
JNK2    human   239 ......SA-- ...K..QPT. .N......K. P.IK..E... .WI..SE..R D.I.T.....
SAPKβ   rat     239 ......CP-- ...K..QPT. .N......K. A......... .S........ ..........
SAPKγ   rat     239 ......CP-- ...K..QPT. ........K. A......... .......... ..........
HOG1    yeast   231 DL..S.-PKD VINTICSENT LKF.TSL.HR DPIP.SER.K --TV------ ----EPD.V.

JNKa    carp    297 LLSKMLVIDA SKRISVEEAL QHPYINVWYD PA-EVEAPPP VITDKQLDER EHTVEEWKEL
JNKb    carp    297 .......... ......D... .......... ..-....... L.I..... ..........
JNK1    human   297 .......... ......D... .......... .S-.A..... K.P..... ...I......
JNK2    human   297 .........P D.....D... R....T.... ..-.A..... Q.Y.A..E.. ..AI......
SAPKβ   rat     297 .........P A.....DD.. .......... ..-....... Q.Y..... ...I......
SAPKγ   rat     297 .......... ......D... .......... .S-.A..... K.P..... ...I......
HOG1    yeast   278 ..E....F.P K...TAAD.. A...SAPYH. .TD.P--VAD AKF.WHFNDA DLPVDT.RVM

JNKa    carp    356 IYKEVLDWEE RMKNGAIRGQ PSPLGAAVIN GSPQPSSSSS INDVSSMSTE PTVASDTDSS
JNKb    carp    356 .F........ .....V.... ...I...... .......... .......... ..........
JNK1    human   356 .....M.L.. .T...V.... ....-.Q.QQ *
JNK2    human   356 .....M.... .S...VVKD. ..--D...-S SNAT..Q... ...I...... Q.L.......
SAPKβ   rat     356 .....MNS.. KT...VVK.. ...S....-. S.ESLPP... V..I.....D Q.L.......
SAPKγ   rat     356 .....M.L.. .T...V.... .......... ..QH.V..P. V..M.....D ..L...*
HOG1    yeast   336 M.S.I..FHK IGGSDGQIDI SATFDDQ.AA ATAAAAQAQA QAQAQVQLNM AAHSHNGAGT

JNKa    carp    416 LEASAGPLSC CR*
JNKb    carp    416 .......... ..*
JNK2    human   413 .D..T...EG ..*
SAPKβ   rat     415 ........G. ..*
HOG1    yeast   396 TGNDHSDIAG GNKGQRSCSC K*
```

Fig.2-4 Comparison of Amino Acid Sequences of cJNKa and cJNKb with Those of Human, Rat, and Yeast MAP Kinases.

The sequences comprise his results, and those reported for human JNK1[7] and JNK2,[8, 9] rat SAPKβ and γ,[10] and yeast HOG1.[11] Identical residues are denoted by periods, and gaps introduced into the sequences are depicted by hyphens. The position of the conservative Thr-Pro-Tyr motif is indicated by shadowing.

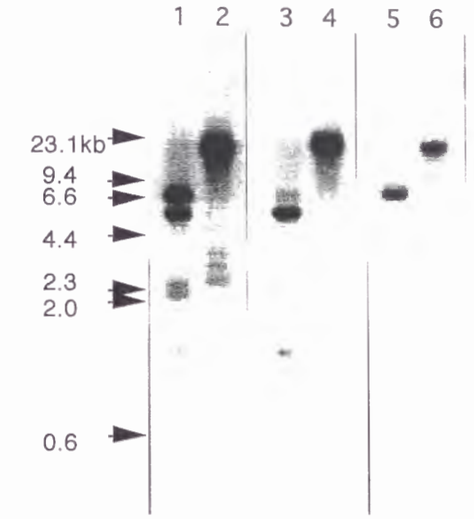

Fig.2-5 Southern Blot Analysis of Carp Genomic DNA.

Carp genomic DNA was digested with *Hin*dIII (lane 1, 3, 5) and *Eco*RI (lane 2, 4, 6), separated by electrophoresis on a 0.8% agarose gel, and transferred onto a nylon membrane. The blots on lanes 3-4 and lanes 5-6 were then hybridized to ^{32}P-labeled cJNKa and cJNKb fragments of the 3'-untranslated region, respectively (see Fig. 2-2). The membranes were washed at 68℃ in 2×SSC containing 0.5% SDS. A mixed probe of cJNKa and cJNKb fragments was used for lanes 1-2. The positions of DNA size markers are indicated.

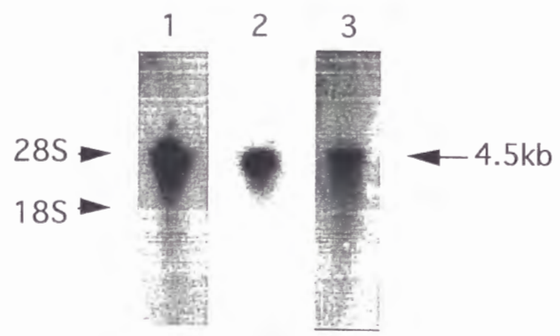

Fig. 2-6 Analysis of cJNKa and cJNKb mRNAs in the Ovary.

Poly $(A)^+$ RNA from the ovary (1 μg) was separated by 1% agarose-formaldehyde gel electrophoresis, transferred onto a nylon membrane and fixed. The blots on lane 2 and lane 3 were then hybridized to ^{32}P-labeled cJNKa and cJNKb fragments of the 3'-untranslated region, respectively (see Fig. 2-2). The membranes were washed at 68℃ in 2×SSC containing 0.5% SDS. A mixed probe of cJNKa and cJNKb fragments was used for lane 1. The molecular size markers consist of carp ribosomal RNAs, indicated by arrowheads.

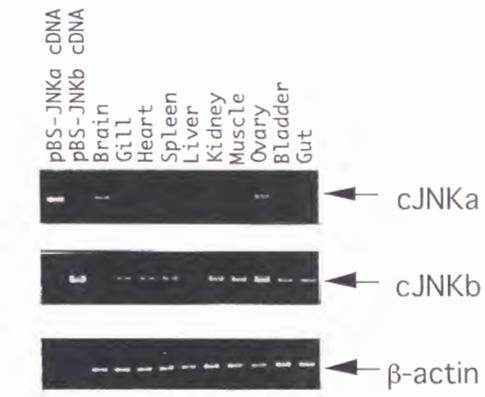

Fig. 2-7 RT-PCR Assay for Detecting cJNKa and cJNKb mRNAs in Various Tissues.

The RNA samples prepared from the brain , gill, heart, spleen, liver, kidney, muscle, ovary, gall bladder, and gut were subjected to RT-PCR assays. As an internal control, the expression of β-actin is shown at the bottom. The full-length cDNAs of cJNKa and cJNKb in pBluescript II were used as templates in PCR as a positive or negative control. All the RNA samples showed no signal when subjected to the reaction without reverse transcriptase. Similar results were obtained in three other experiments.

Section 2
Activation of cJNKa and cJNKb by Environmental Stresses

In mammalian cells, it has been shown that the JNK/SAPK MAP kinase pathway is activated by a variety of extracellular stimuli including osmotic stress, UV light, heat shock and inflammatory cytokines.[2, 21, 13] The author examined whether the isolated cJNKa and cJNKb were activated by these environmental stresses, and also whether any difference in activation pattern was seen between cJNKa and cJNKb.

Materials and Methods

Materials

TNFα, IL-1, and EGF were kindly gifted by Dr. E. Nishimura.

Oligonucleotides

Oligonucleotides used for PCR primers are: cJNK-5N, 5'-CAGGCCATGGCAATGAACAAAAATAAGCGAGA-3' (sense) and cJNK-3B, 5'-CGGGATCCTCATCTGCAGCAGCTCAGGGGTCC-3' (antisense) to amplify the ORF of cJNKa and cJNKb.

DNA Constructs

To obtain pcDNA3 HA-cJNKa and pcDNA3 HA-cJNKb, the ORF of cJNKa or cJNKb was cloned into pcDNA3 HA vector at the *Nco*I/*Bam*HI sites. pGEX-3X-cJun (1-79) for production of GST-cJun (1-79) was kindly gifted by Dr. M. Hibi.[6]

Preparation of Recombinant Proteins

GST-cJun (1-79) was bacterially expressed and purified on glutathione-Sepharose (Pharmacia). The protein concentration was determined by the Bradford assay (Bio-Rad).

Transfection

The method of calcium phosphate transfection was employed. EPC was inoculated at a density of 2×10^6/60-mm dish and cultured at 30℃ in Eagle's minimum essential medium (MEM) supplemented with 10% FBS

for 24 h until transfection. Ten micrograms of pcDNA3 HA-cJNKa or cJNKb were used to form a precipitate containing calcium phosphate and DNA. At 6 h after the transfection, the cells were treated with 10% glycerol in PBS. After an additional 72 h incubation in MEM supplemented with 10% FBS, the cells were exposed to various stresses.

Immunoprecipitation

The cells were solubilized with lysis buffer (20 mM Tris-HCl (pH 7.4), 1% Triton X-100, 5 mM EGTA, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM pyrophosphate, 5 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride 10 μg/ml leupeptin, 2 mM dithiothreitol) and centrifuged at $15,000\times g$ for 15 min at 4℃. The HA-tagged protein kinases were immunoprecipitated by incubation 1 h at 4℃ with anti-HA antibodies (12CA5) pre-bound to protein G Sepharose (Pharmacia).

Protein Phosphorylation

Kinase assays were performed using immunoprecipitates of cJNKa and cJNKb. The immunecomplexes were washed with kinase buffer (50 mM HEPES (pH 7.4), 10 mM β-glycerophosphate, 10 mM $MgCl_2$, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 1 mM dithiothreitol). The assays were initiated by the addition of 3 μg GST-cJun (1-79), 50 μM ATP, and 3 μCi [γ-^{32}P] ATP in a final volume of 20 μl of kinase buffer. The reactions were terminated after 15 min at 30℃ by adding Laemmli sample buffer. The phosphorylation of the substrate protein was examined after SDS-PAGE by autoradiography.

Results and Discussion

Activation of cJNKa and cJNKb by Various Stresses

To characterize the activation of cJNKa and cJNKb, the author constructed an expression vector harboring HA-tagged cJNKa or cJNKb that could be immunoprecipitated using anti-HA antibodies. The protein kinase activity was measured by an immunecomplex kinase assay with the substrate GST-cJun (1-79), which is known to be a good substrate for mammalian JNK.[6] Exposure of the transfected EPC to UV light, hypertonic stress, and heat shock caused activation of both cJNKa and cJNKb that was

evident at 30 min after the exposure (Fig. 2-8a). However, activation of cJNKa or cJNKb was not detected in the cells treated with TNFα and IL-1 (Fig. 2-8a), which have been shown to activate mammalian JNKs.[13] The failure of these proinflammatory cytokines to activate cJNKs might be due to the use of human cytokines to fish cells. Examination of dose response revealed that activation of both of cJNKa and cJNKb by UV irradiation and hypertonic stress (Sorbitol supplementation) occurred in a dose dependent manner (Figs. 2-8a and 2-8b).

Time Course of Activation of cJNKs

Time course of cJNKa and cJNKb activation was examined at a dose of 750 mM sorbitol supplementation (hypertonic stress) or of 80 J/m^2 (UV irradiation). As a result, activation of both cJNKa and cJNKb induced by the hypertonic stress became detectable at 15 min after exposure to the stress, with maximal activation at 60 min followed by a progressive decline in kinase activity at later times (Fig. 2-9a). In the case of UV irradiation, activation of cJNKa and cJNKb was observed in a similar manner, with maximal activation at 60 min (Fig. 2-9b). However, the UV-induced activation of cJNKs was sustained for 120 min at a relatively high level. Treatment of EPC with heat shock (40℃) caused activation of cJNKs in an increasing manner until 30 min after the treatment, although at later times kinase activity could not be measured because of detachment of the cells from the dish (Fig. 2-9c).

These results suggest that The JNK/SAPK MAP kinase pathway in which cJNKs participate functions to transduce the environmental stress signals in EPC and that no significant difference between cJNKa and cJNKb was seen in activation pattern in response to the environmental stresses examined.

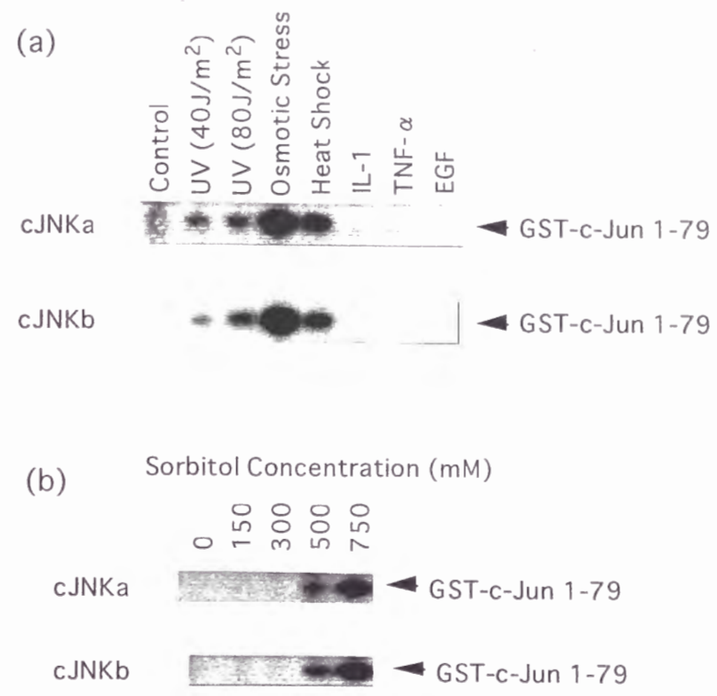

Fig. 2-8 Activation of cJNKa and cJNKb by Various Stresses.

(a) EPC was transfected with a plasmid harboring HA-cJNKa or HA-cJNKb cDNA and cultured for 72 h. The cells were exposed to UV light (40 or 80 J/m^2), hypertonic stress (750 mM sorbitol), heat shock (30→40℃), IL-1 (10 ng/ml), TNFα (10 ng/ml), or EGF (10 ng/ml). After 30 min, the cells were harvested, and activity of the HA-tagged proteins was measured by an immunecomplex kinase assay using [γ-^{32}P] ATP and GST-cJun (1-79) as substrates. 'Control' shows a result of the cells without exposure to stress.

(b) The transfected cells were treated with 150-750 mM sorbitol for 30 min. Activity of the HA-tagged proteins was measured as described in (a). 'Control' shows a result of the cells without sorbitol treatment.

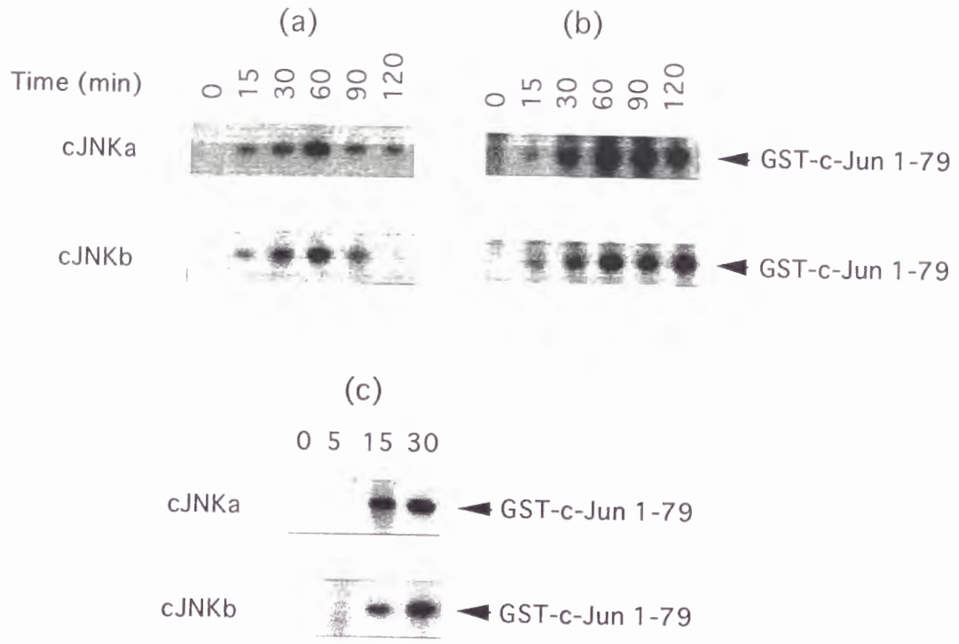

Fig. 2-9 Time Course of Activation of cJNKs.

EPC was transfected with a plasmid harboring HA-cJNKa or HA-cJNKb cDNA and cultured for 72 h. The cells were exposed to hypertonic stress (750 mM sorbitol)(a), UV light (80 J/m^2)(b), or heat shock (30→40℃)(c). After 0, (5), 15, 30, 60, 90 min, the cells were harvested, and activity of the HA-tagged proteins was measured by an immunecomplex kinase assay using [γ-^{32}P] ATP and GST-cJun (1-79) as substrates. Activity was not determined for the cells exposed to heat shock for 60 and 90 min, because heat shock (beyond 30 min) leaded to a cell detachment from dish.

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CHAPTER III

The p38 MAP Kinase Pathway in Carp

Section 1
Structure and Expression of Carp p38s (cp38a and cp38b)

The p38 MAP kinase pathway as well as The JNK/SAPK MAP kinase pathway is the newly identified signal transduction pathway homologous to the classical MAP kinase pathway in their overall form.[1-6] Different from the classical MAP kinase pathway, however, the p38 pathway appears to be activated by a wide variety of cellular stresses including osmotic shock, UV light, heat shock, lipopolysaccharides (LPS), and inflammatory cytokines, like the JNK/SAPK pathway.[6, 7] p38 is known to be activated through its phosphorylation on Thr and Tyr residues at the Thr-Gly-Tyr dual phosphorylation motif.[6, 7] As in the classical MAP kinase pathway,[8-10] the nuclear translocation of activated p38 seems to be a crucial step for regulatory features of the p38 MAP kinase cascade on gene expression.[7]

To date, the cDNAs for p38 have been isolated from man,[11-15] mouse,[16] frog,[17] and yeast.[18] Here the author reports the molecular cloning of two p38 (cp38a and cp38b) cDNAs from carp. Tissue differences in expression of cp38a and cp38b were also examined.

Materials and Methods

PCR Cloning of cp38 cDNA Fragment

The cDNA fragment encoding a partial sequence of carp p38 homologous to mammalian p38 was successfully obtained by the same procedure described in Chapter II and used to screen the carp ovary cDNA library.

Preparation of Probes for Southern and Northern Blot Analyses

5'- and 3' probes of cp38a were prepared from the cDNA fragments generated by digestion of the plasmid containing the full-length cp38a cDNA (cloned at *Eco*RI/*Xho*I sites) with *Eco*RI/*Eco*RV and *Hin*dIII/*Xho*I, respectively.

5'- and 3' probes of cp38b were prepared from an *Eco*RI/*Bam*HI and an*Sca*I/*Xho*I cDNA fragments of the plasmid containing the full-length cp38b cDNA (cloned at *Eco*RI/*Xho*I sites), respectively. ORF probes of cp38a and cp38b were prepared by PCR amplification of the ORF of cp38a and cp38b cDNAs. ^{32}P-labelling procedure was followed by the method described in Chapter II.

Results and Discussion

cDNA Cloning of cp38a and cp38b

PCR cloning of carp p38 cDNA fragment successfully yielded an expected size of DNA corresponding to a potential p38. In fact, a homology search with the GenBank database revealed that the cDNA fragment was homologous to the inner part of mouse p38 cDNA.

The author first examined the tissue distribution of p38 mRNA in carp by Northern blot analysis with the PCR fragment as a probe. As a result, expression of 4 kb mRNA was detected in all the tissues examined (Fig. 3-1). An additional mRNA (3.2 kb) was observed at a high level in the ovary. In order to isolate the full length cDNA for carp p38, the author screened the cDNA library constructed from ovary RNA (see Chapter II).

The cDNA library was screened with the PCR fragment as a probe. Screening of approximately 3×10^5 plaques with the PCR fragment as a probe yielded 7 positive clones. Restriction mapping and sequence analysis of both the 5'- and 3'-ends of the clones showed the presence of two distinct carp p38s (cp38a and cp38b) (Figs. 3-2). The longest clones exhibiting different restriction maps were subjected to determination of the complete nucleotide sequence. The amino acid sequences predicted by the nucleotide sequence of cp38a (DDBJ accession number D83274, Fig. 3-3a) and cp38b (Fig. 3-3b) both contained 361 residues with a predicted mass of 41.7 kDa and 41.5 kDa, respectively. The in-frame stop codons in the 5' and 3' regions of the cDNAs indicated that these clones contain the entire carp p38 coding region (Figs. 3-3a and 3-3b).

Multiple alignments of amino acid sequences of cp38a and cp38b with those of other animals are shown in Fig. 3-4. Comparison with the protein sequences in the SWISS-PROT database by m-align indicated that cp38a is 85%, 72%, 62%, 83%, and 50%, and cp38b is 86%, 69%, 61%, 83%,

and 50%, identical in primary structure to human p38α,[11, 12] p38β,[13] and p38γ,[14] frog Mpk2,[17] and yeast HOG1,[18] respectively. When the sequence of cp38a is compared with that of cp38b, 92.0% of the residues are identical; among 361 residues in the deduced sequence, 29 residues are different. MAP kinases are known to be activated through dual phosphorylation of adjacent threonine (Thr) and tyrosine (Tyr) residues. These putative phosphorylation sites are Thr-181 and Tyr-183 in both of cp38a and cp38b. These sites possess the Thr-Gly-Tyr tripeptide motif, which is conserved in the p38 subgroup of the MAP kinase family. Both of cp38a and cp38b are most homologous to human p38α and frog Mpk2 in overall structure. These similarities suggest that cp38a and cp38b belong to the p38 subgroup of the MAP kinase superfamily.

Difference in Tissue Distribution between cp38a and cp38b

The cDNA fragment isolated by PCR cloning possibly probed both cp38a and cp38b mRNAs in the Northern blot analysis of various tissues (Fig. 3-1), because of the sequence similarity in the inner part of the ORFs of the cloned cp38a and cp38b cDNAs. Actually, multiple bands were observed by Southern blot analysis of carp genomic DNA using the fragment as a probe, indicating that the probe hybridized to both cp38a and cp38b genes (data not shown). In order to clarify whether there was a difference in length between cp38a and cp38b mRNAs, by using 5'-, 3'-, and ORF sequences of cp38a and cp38b cDNAs (Fig. 3-2) as a probe, the author carried out Northern blot analysis with poly (A) rich RNA of the ovary, where two lengths of the transcript, 4 kb and 3.2 kb, were detected (Fig. 3-1). The specificity of these probes was confirmed by a genomic Southern blot analysis to distinguish cp38a and cp38b mRNAs (Fig. 3-5b). As a result, all of the three probes of cp38a recognized the 3.2 kb mRNA, while all for cp38b probed the 4 kb mRNA (Fig. 3-5a). These facts indicate that the transcript of cp38a is 3.2 kb and that of cp38b is 4 kb in length. In consistent with the result described above (Fig. 3-1), Northern blot analysis with the probes specifically recognizing cp38a revealed cp38a mRNA was predominantly expressed in the ovary (Fig. 3-5a). Abundant expression of mRNA in the ovary has not been observed for p38α, p38β, p38γ, and p38δ in the mammalian p38 group, although expression of p38 γ mRNA is predominant in the skeletal muscle. The predominant expression of cp38a mRNA and the

abundant expression of cp38b mRNA imply an important role of the p38 MAP kinase pathway in the ovary of carp, or in embryos as a maternal factor like the JNK/SAPK MAP kinase pathway (see Chapter II).

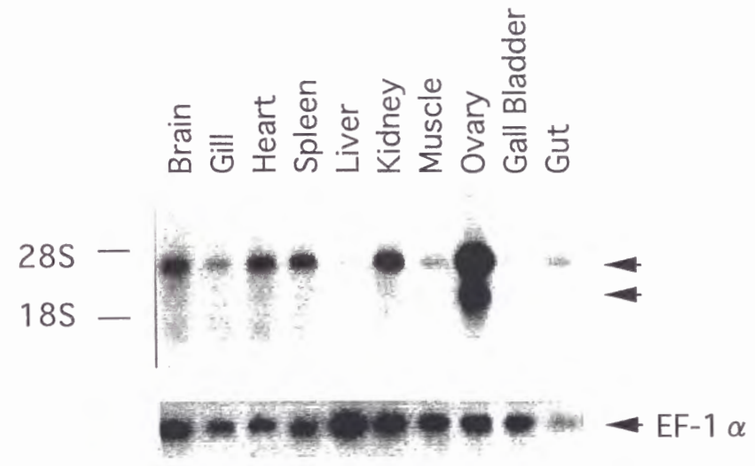

Fig. 3-1 Tissue Distribution of Carp p38 mRNA.

Poly $(A)^+$ RNA samples were prepared from the brain, gill, heart, spleen, liver, kidney, muscle, ovary, gall bladder, and gut. Each RNA sample (2 μg) was separated by 1% agarose-formaldehyde gel electrophoresis, transferred onto a nylon membrane and fixed. The p38 cDNA fragment derived by RT-PCR cloning was used as a probe. After hybridization, the membrane was washed at 65℃ in 2×SSC containing 0.5% SDS. The blot was reprobed with medaka elongation factor-1α cDNA as an internal control. The positions of 28S and 18S rRNAs are indicated on the left by arrowheads.

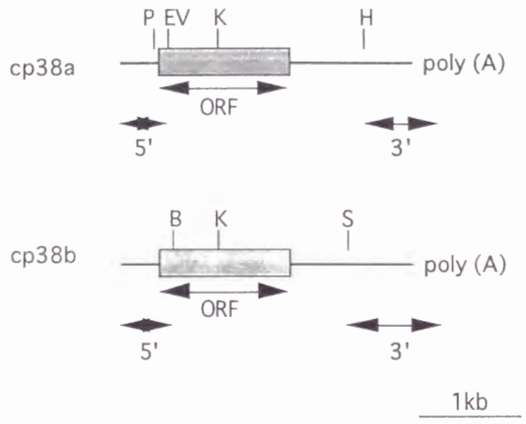

Fig. 3-2 Restriction Map of the cDNA Clones of cp38a and cp38b.

Filled boxes indicate the ORFs. Restriction endonuclease cleavage sites are indicated as follows: B, *Bam*HI; H, EV, *Eco*RV; *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sca*I. The positions of the 5'-, 3'-, and ORF probes used for Southern and Northern blot analyses are illustrated by a line with double-arrowheads.


```
-184                                                                GGCT  -181
-180 GGCATGAACTAAACACATCGATGTGGTTCGGTCTTTAGTACCCGTGTGCACGCTCGACTTTATCGGTCGTTTACTATCCATTTTTGATTG  -91
 -90 TTGCACGAAAACTCAACCGCCTTGAATCACAGGCAGGCAAGCCAAGGCAAGCAAAAGTCTCAACTCGCCTCGGGCGCTCGTTCTGCAGGC   -1

   1 ATGTCGCAGAAAGAAAGACCCACTTTCCATCGACAGGAGGTCAACAAGACAATATGGGAGGTCCCTGTGCGATATCAGAACTTGTCTCCC   90
   1 M  S  Q  K  E  R  P  T  F  H  R  Q  E  V  N  K  T  I  W  E  V  P  V  R  Y  Q  N  L  S  P    30

  91 GTCGGCTCTGGTGCATACGGAACTGTTTGCTCTGCATATGATGAAAAGACAGGATTGAAGGTTGCCGTGAAGAAGCTGTCAAGGCCGTTT  180
  31 V  G  S  G  A  Y  G  T  V  C  S  A  Y  D  E  K  T  G  L  K  V  A  V  K  K  L  S  R  P  F    60

 181 CAATCCATCATCCATGCCAAGCGGACCTACAGGGAATTGCGACTACTCAAGCATATGAAACATGAGAATGTGATCGGCCTGCTGGATGTT  270
  61 Q  S  I  I  H  A  K  R  T  Y  R  E  L  R  L  L  K  H  M  K  H  E  N  V  I  G  L  L  D  V    90

 271 TTCACACCTGCCACCAGCCTGGAGGAGTTTAATGATGTGTACCTGGTGACTCATCTAATGGGTGCTGACCTGAATAATATTGTGAAGTGT  360
  91 F  T  P  A  T  S  L  E  E  F  N  D  V  Y  L  V  T  H  L  M  G  A  D  L  N  N  I  V  K  C   120

 361 CAGAAGCTGACCGACGATCATGTTCAGTTCCTTATTTACCAGATCCTGCGAGGGCTTAAGTACATCCATTCGGCTGACATAATTCACAGG  450
 121 Q  K  L  T  D  D  H  V  Q  F  L  I  Y  Q  I  L  R  G  L  K  Y  I  H  S  A  D  I  I  H  R   150

 451 GACTTGAAACCCAGTAATCTGGCAGTAAATGAAGACTGTGAACTTAAAATCCTTGACTTTGGTCTGGCTCGACACACAGATGATGAGATG  540
 151 D  L  K  P  S  N  L  A  V  N  E  D  C  E  L  K  I  L  D  F  G  L  A  R  H  T  D  D  E  M   180

 541 ACAGGGTATGTGGCCACAAGGTGGTACCGTGCTCCAGAGATCATGCTTAACTGGATGCACTACAACATGACAGTGGACATTTGGTCAGTG  630
 181 T  G  Y  V  A  T  R  W  Y  R  A  P  E  I  M  L  N  W  M  H  Y  N  M  T  V  D  I  W  S  V   210

 631 GGCTGCATCATGGCTGAGCTGTTGACAGGCAGGACTCTGTTCCCTGGCACAGATCATATAAACCAGCTTCAACAGATAATGCGACTTACA  720
 211 G  C  I  M  A  E  L  L  T  G  R  T  L  F  P  G  T  D  H  I  N  Q  L  Q  Q  I  M  R  L  T   240

 721 GGAACTCCACCTGCCTCTCTAATAAGCAGGATGCCTAGCCATGAGGCTCGTACTTACATCAACTCGCTTCCTCAGATGCCCAAGAGGAAT  810
 241 G  T  P  P  A  S  L  I  S  R  M  P  S  H  E  A  R  T  Y  I  N  S  L  P  Q  M  P  K  R  N   270

 811 TTTTCTGAAGTGTTTATCGGGGCTAATCCACAAGCTGTGGACCTTTTAGAAAAGATGCTGGTTTTGGACACCGATAAGCGCATCACAGCG  900
 271 F  S  E  V  F  I  G  A  N  P  Q  A  V  D  L  L  E  K  M  L  V  L  D  T  D  K  R  I  T  A   300

 901 GCGGAGGCTCTGGCTCACCCTTACTTTGCTCAGTACCATGACCCAGATGATGAACCCGAGGCAGAGCCTTTCGACCAGAGCTTTGAGAGC  990
 301 A  E  A  L  A  H  P  Y  F  A  Q  Y  H  D  P  D  D  E  P  E  A  E  P  F  D  Q  S  F  E  S   330

 991 CGAGAGCTTGATATTGAAGAGTGGAAACGTCAGACATATGAAGAGGTGATAAGTTTTGAGCCGCCAGTCTTTGATGGAGATGAAATGGAA 1080
 331 R  E  L  D  I  E  E  W  K  R  Q  T  Y  E  E  V  I  S  F  E  P  P  V  F  D  G  D  E  M  E   360

1081 TCTTGACCTTTTCTTCTGGATGGCAATCAAACCAACTGTACTGCTTATACAGTAATGCTGTCATTTTGCAGTATTGTAATCAGCCAGTCA 1170
 361 S  *                                                                                        361

1171 GTACGATGTTTGACTGTTTCTTTTCATATTTTTCATGTCGTCTACTGTGCCAAACAGGATAGAAGTCATAGAAAATATTATTTTACACAT 1260
1261 TGAATCTCTTCAAAAGAATCTGATGGTAATAATATCAACAACTATTTTTTAACAGTTTGCTTTGGTCAGTTCCAAGATATTCATATATGA 1350
1351 CAGTCATGCAAGTCTACCTGTAAATGTAATATCTCCCTGAAATTTCGTAGTATTTAGTTGTGCTGTTCTTTCTGTGTACAATGCGTCACA 1440
1441 TTTGCAGTATTTTTAAAATGTTCAGAAAACATTCTTCATTTAGTTTCTGTTAATATATATATTTTTCCAATCAAATAATGAGCATTGTAT 1530
1531 TTAACGATTTACAGTGTTCCTCAATCAGTTTTCATAAAGATGGAATTTTTTGGAGGCTCCTTTGATAGAGCTAGGATGAAAAATGTAAAT 1620
1621 TGTTGCATTGGATTTCGGTGATAAGTTTAGGCTATTTGTAAATTATTCTTTTCATAAAGCTTTTGGAATCTGTAGCTGTTTTATTTCTGA 1710
1711 TCTTTTTCACATTGTTTGTGTATGTTTCATAGGGAGAGAAAGTGTGAGGTTGTGTGTGTGTGCGTGTGTGTCCACCTGAGGTTGCTACTA 1800
1801 TGAACTATATTCGATCTGATTCTGCCTCCAGTAGCTCTTTTTAAAATGCTATTAATTGAATGTTCAAATATGAAAGCAGAGCAAGATATT 1890
1891 TGTACTGTTGAGGTGGTTTTATTTATGAACCTGAAAAGCTGCAACTCCCTTAGCACCTGACAGAAAACACAGAATCAAACACACCATTGT 1980
1981 TTACTGAAAATAAAGGCCGATATTTTTCAGAAAAAAAAAAAAAAAA                                             2026
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Fig. 3-3 (a) Nucleotide and Deduced Amino Acid Sequences of cp38a.

The open reading frame (361 amino acids) is preceded by two in-frame stop codons, indicated by underlining. The putative dual-phosphorylation site is double-underlined. The predicted stop codon is denoted by an asterisk.

```
-174       CAGAGCTGGAGCTTGTGCACGATCTTCGGCGCTCGCGCCCATGCATTTGACCGATGCCATGACTGGACTGTGAACGCGGAGCTT   -91
-90 GCGCTTTTGTTTAGTTATTTTTTGTTGGTTTCTATCAAGCACTGAGATCAAGAGAGCAGCACAGAAGTTTCCACTCCTCTAACTACAGGA   -1

  1 ATGTCCCACAAGGAGAGAGACCGACTTTCTATCGACAGGAGCTCAATAAGACCATATGGGAGGTGCCGGAGCGCTACCAGAACTTGTCGCCC   90
  1 M  S  H  K  E  R  P  T  F  Y  R  Q  E  L  N  K  T  I  W  E  V  P  E  R  Y  Q  N  L  S  P   30

 91 GTGGGGTCTGGAGCTTACGGATCCGTGTGCTCCGCGTTGGACACAAAGTCAGGCCTGAGGGTCGCCGTCAAGAAGCTGTCCCGGCCGTTC   180
 31 V  G  S  G  A  Y  G  S  V  C  S  A  L  D  T  K  S  G  L  R  V  A  V  K  K  L  S  R  P  F    60

181 CAGTCCATGATCCACGCCAAGCGCACCTACAGAGAACTGCGGCTCCTCAAACACATGAAACACGAGAATGTAATTGGTCTACTAGATGCT   270
 61 Q  S  M  I  H  A  K  R  T  Y  R  E  L  R  L  L  K  H  M  K  H  E  N  V  I  G  L  L  D  A    90

271 TTCTCGCCCGCTACCTGTCTAGCAGGATTCAATGACGTGTATCTGGTGACCCACCTCATGGGAGCAGACCTCAATAATATCGTCAAGTGC   360
 91 F  S  P  A  T  C  L  A  G  F  N  D  V  Y  L  V  T  H  L  M  G  A  D  L  N  N  I  V  K  C   120

361 CAGAAGCTGACGGATGATCACGTCCAGTTCCTCATTTATCAGATCCTGCGAGGACTGAAGTATATTCACTCAGCAGACATCATCCACAGA   450
121 Q  K  L  T  D  D  H  V  Q  F  L  I  Y  Q  I  L  R  G  L  K  Y  I  H  S  A  D  I  I  H  R   150

451 GATCTTAAACCCAGTAACTTGGCTGTAAATGAAGACTGTGAACTTAAGATTCTGGACTTTGGGCTGGCCCGGCTGACTGATGATGAAATG   540
151 D  L  K  P  S  N  L  A  V  N  E  D  C  E  L  K  I  L  D  F  G  L  A  R  L  T  D  D  E  M   180

541 ACGGGATACGTTGCCACCCGATGGTACCGCGCCCCAGAGATCATGCTCAACTGGATGCACTACAACATGACAGTGGACATCTGGTCGGTG   630
181 T  G  Y  V  A  T  R  W  Y  R  A  P  E  I  M  L  N  W  M  H  Y  N  M  T  V  D  I  W  S  V   210

631 GGCTGCATAATGGCTGAGCTCCTCACAGGACGGACCCTGTTTCCCGGCACTGATCATATAAACCAGCTTCAGCAGATAATGCGACTGACG   720
211 G  C  I  M  A  E  L  L  T  G  R  T  L  F  P  G  T  D  H  I  N  Q  L  Q  Q  I  M  R  L  T   240

721 GGAACCCCGCCGGCCTCTCTAATAAGCAGGATGCCGAGCCACGAGGCCAGGAACTACATCAATTCTCTTTCTTATATGCCCAAGAGGAAC   810
241 G  T  P  P  A  S  L  I  S  R  M  P  S  H  E  A  R  N  Y  I  N  S  L  S  Y  M  P  K  R  N   270

811 TTCGCAGATGTGTTTGTTGGTGCAAACCCAATGGCTGTGGACCTGCTGGAGAAGATGCTGGTTCTGGACACAGATAAACGGATAACTGCG   900
271 F  A  D  V  F  V  G  A  N  P  M  A  V  D  L  L  E  K  M  L  V  L  D  T  D  K  R  I  T  A   300

901 TCACAGGCTCTGGCGCACCCGTACTTCGCCCAGTACCACGACCCGGACGACGAGCCCGAGGCAGACCCCTACGACCAGAGCTTCGAGAGC   990
301 S  Q  A  L  A  H  P  Y  F  A  Q  Y  H  D  P  D  D  E  P  E  A  D  P  Y  D  Q  S  F  E  S   330

991 CGCGATCTGGACATCGAAGAGTGGAAACGGCTCACGTATGAAGAGGTGATCAGCTTCGAGCCTCCCGTGTTCGACGGAGACGAGATGGAG  1080
331 R  D  L  D  I  E  E  W  K  R  L  T  Y  E  E  V  I  S  F  E  P  P  V  F  D  G  D  E  M  E   360

1081 TCATGAAGGCTGCTCCACCTCCAGGACCTTCATCCAGACAGAAAAATGACATTCAAGTGCCTGCCATCATCAAGTGCTGGCTTCCCCGTG  1170
 361 S  *                                                                                        362

1171 TTCTGAGACTGCTTATCCATGCCTTCATACATGCTCTGTTAAATAAGGGGGGAACAAAAACGTGCAAACTTTTAAAAAAGGACACAGAGA  1260
1261 ATGTAAGGTTTTGTGTCCGGTGCCCCAGTAACTGTTTGGTCACTCGTACACAATTAGAACAACTTGAGGGTGAGAAAATGACCACATTTT  1350
1351 CATTTTTGGGCGAACTATCCCTTTAACTACTTTTTTGGACAGAACACAAAAAAACCAATCAGTCAAATTCAAAACAGCATTTTAAGCTCA  1440
1441 AAGTTTCATATTCTTTCTCTGGGGAAGCAAAACATTTACCTCCGGACTAATCTTGTAGCCTGCACAATAAATAGAGGCCAGAAAAACTCA  1530
1531 AGTACTGTCAGAATACTAAGTTTTAGATATGTAAATAAAATGGCACTNCTGTACATAAATGATAACAATGTGCACCAACAGTTCTCTTTA  1620
1621 GTGTTTGCANCAAAGCACGCATNATAAACANCAAATGANCGAGCTAAAGAGTGAACCCTGTCACCTAAAGAGAATGTAACTATTATTTAG  1710
1711 TGCTCTGTGTAGAATGTAAACAGAGGAGTATATATAGACGTAGTGTTCTTCTGCCAACTGCATCTCTGTCTCGGACAATAATAGATGACA  1800
1801 CAAACAAAATTCATGCGGTTTACATGAATGAATGCAGATATGCTTGTTCTGAAAATGTATTGGGAAATATTTAGAGAAAGAAAAAAAAAA  1890
1891 TGTGGTGGTCAGTGCTAAAGTGAGAATGTTAAACTTTTTTTTACATATAAAATATTATGGGCGAATCTCACGAAACCTCTTCAGAACATG  1980
1981 GCCAGATCATAATGAACTACAAAATCAGAAGAACATTTTTTTTTTTTTGTTAATGTATTTTAAATATAAAGAAAAATATTTTGCATAAAG  2070
2071 AAAGTCAATAATTACGTTTTAACAATCAGTAATTGTTTTTATATTATTATTTAGATATTAATGATATATTCCATACTTACATATATTACC  2060
2161 CTATAATGCAAAAAAAAAAAAAAAAAAA                                                               2189
```

Fig. 3-3 (b) Nucleotide and Deduced Amino Acid Sequences of cp38b.

The open reading frame (361 amino acids) is preceded by a in-frame stop codon, indicated by underlining. The putative dual-phosphorylation site is double-underlined. The predicted stop codon is denoted by an asterisk.


```
p38a    carp      1 MS---QKERP TFHRQEVNKT IWEVPVRYQN LSPVGSGAYG TVCSAYDEKT GLKVAVKKLS
p38b    carp      1 ..----H.... ..Y...L... .....E.... .......... S....L.T.S ..R.......
p38α    human     1 ..---.-... ..Y...L... .....E.... .......... S..A.F.T.. ..R.......
p38β    human     1 ..---G-P.A G.Y...L... V....Q.L.G .R........ S......ARL RQR.......
p38γ    human     1 ..SPPPA... ..Y..VT... A...RAV.RD .Q........ A....V.GR. .A...I...Y
Mpk2    frog      1 ..---SNQSY V.Y...L... L....D.... .T........ S...SF.TR. A.RI......
HOG1    yeast     1      MTTNE E.I.TQIFG. VF.ITN..ND .N...M..F. L....T.TL. SQP..I..IM

p38a    carp     58 RPFQSIIHAK RTYRELRLLK HMKHENVIGL LDVFTPATSL EEFNDVYLVT HLMGADLNNI
p38b    carp     58 .....M.... .......... .......... ..A.S...C. AG........ ..........
p38α    human    57 .......... .......... .......... .......R.. .......... ..........
p38β    human    57 .....L...R .......... .L........ .........I .D.SE..... T.........
p38γ    human    61 .....ELF.. .A........ ..R....... ......DET. DD.T.F...M PF..T..GKL
Mpk2    frog     58 .......... .......... .......... ....S..K.F .......... ..........
HOG1    yeast    57 K..STAVL.. ......K... .LR...L.C. Q.I.-----. SPLE.I.F.. E.Q.T..HRL

p38a    carp    118 VKCQKLT--- -----DDHVQ FLIYQILRGL KYIHSADIIH RDLKPSNLAV NEDCELKILD
p38b    carp    118 .......--- -----..... .......... .......... .......... ..........
p38α    human   117 .......--- -----..... .......... .......... .......... ..........
p38β    human   117 ....AGAHQG ARLAL.E... ..V..L.... ......G... .......V.. .....R....
p38γ    human   121 M.HE..G--- -----E.RI. ..V..M.K.. R...A.G... .....G.... ..........
Mpk2    frog    118 .......--- -----..... .......... ......G... .......... ..........
HOG1    yeast   117 LQTRP.E--- -----KQF.. YFL....... ..V...GV.. .......ILI ..N.D...C.

p38a    carp    170 FGLARHTDDE MTGYVATRWY RAPEIMLNWM HYNMTVDIWS VGCIMAELLT GRTLFPGTDH
p38b    carp    170 .....L.... .......... .......... .......... .......... ..........
p38α    human   169 .......... .......... .......... ...Q...... .......... ..........
p38β    human   177 .....QA.E. .......... .......... ...Q...... ..........Q .KA....S.Y
p38γ    human   173 .....QA.S. .....V.... ....VI.... R.TQ...... .......MI. .K...K....
Mpk2    frog    170 ........E. .......... .......... ...Q...... .......... ..........
HOG1    yeast   169 .....IQ.PQ .....S..Y. .......T.Q K.DVE..... A...F..MIE .KP....K..

p38a    carp    230 INQLQQIMRL TGTPPASLIS RMPSHEARTY INSLPQMPKR NFSEVFIGAN PQAVDLLEKM
p38b    carp    230 .......... .......... ........N. ....SY.... ..AD..V... .M........
p38α    human   229 .D..KL.L.. V...G.E.LK KIS.ES..N. .Q..T....M ..AN...... .L........
p38β    human   237 .D..KR..EV V...SPEVLA KIS.EH.... .Q...P..QK DL.SI.R... .L.I...GR.
p38γ    human   233 LD..KE..KV ......EFVQ .LQ.D..KN. MKG..ELE.K D.ASILTN.S .L..N.....
Mpk2    frog    230 .D..KL.L.. V...EPE.LQ KIS.EA..N. .Q...Y...M ..ED..L... ..........
HOG1    yeast   229 VH.FSI.TD. L.S..KDV.N TIC.ENTLKF VT...HRDPI P...R.KTVE .D........

p38a    carp    290 LVLDTDKRIT AAEALAHPYF AQYHDPDDEP EAEPFDQSFE SRELDIEEWK RQTYEEVISF
p38b    carp    290 .......... .SQ....... .......... ..D.Y..... ..D....... .L........
p38α    human   289 ....S..... ..Q....A.. .......... V.D.Y..... ..D.L.D... SL..D.....
p38β    human   297 ....S.Q.VS .......A.. S.....E... ....Y.E.V. AK.RTL.... EL..Q..L..
p38γ    human   293 ....AEQ.V. .GQ....... ESL..TE... QVQKY.D..D DVDRTLD... .V..K..L..
Mpk2    frog    390 .......... .......S.. .......... I...Y..... .......... .L.....TC.
HOG1    yeast   289 ..F.PK.... ..D......S .P....T... V.DAKFDWHF NDADLPVDTW .VMMYSE.LD

p38a    carp    350 EPPVFDGDEM ES*
p38b    carp    350 .......... ..*
p38α    human   349 V..PL.QE.. ..*
p38β    human   357 K..EPPKPPG SLEIEQ*
p38γ    human   353 K..RQL.ARV SKETPL*
Mpk2    frog    350 V..OL.SE.. ..*
HOG1    yeast   349 FHKIGGS.GQ IDISATFDDQ V>
```

Fig. 3-4 Comparison of Amino Acid Sequences of cp38a and cp38b with Human, Frog, and Yeast MAP Kinases.

The sequences comprise our results, and those of human p38α,[11,12] p38β,[13] and p38γ,[14] frog Mpk2,[17] and yeast HOG1,[18]. Identical residues are denoted by periods, and gaps introduced into the sequences are depicted by hyphens. The position of the conservative Thr-Gly-Tyr motif is indicated by shadowing.

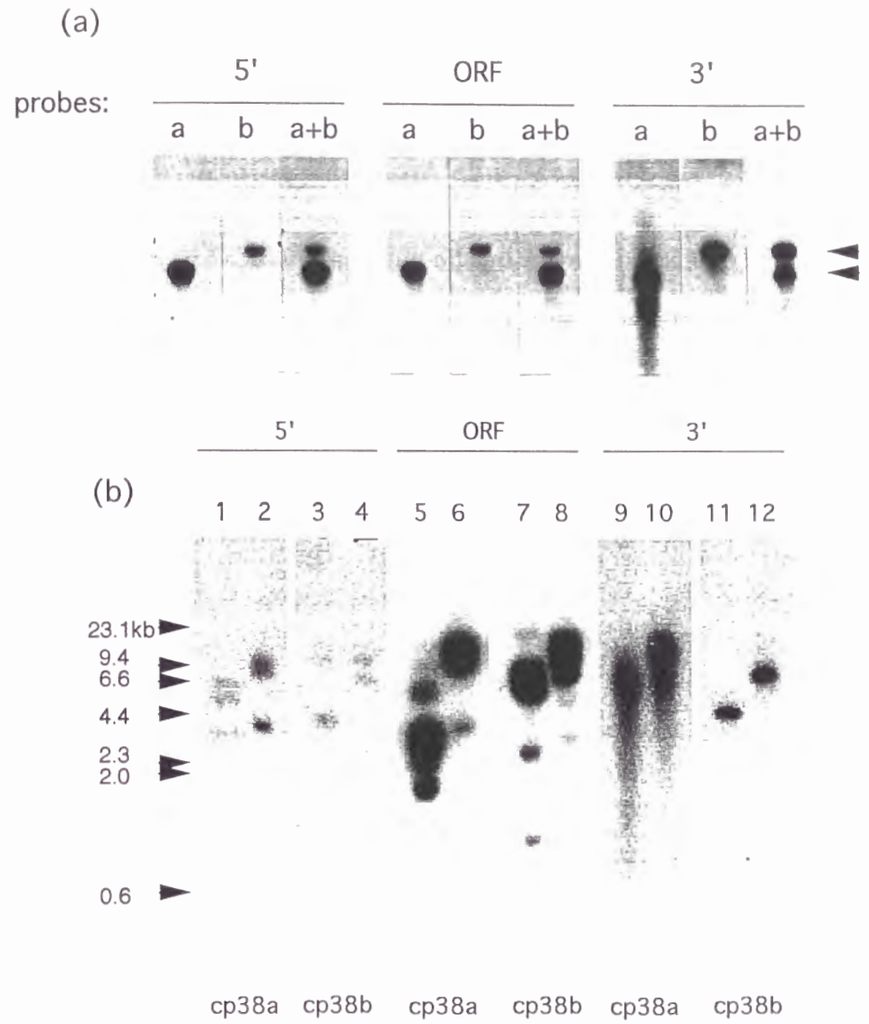

Fig. 3-5 (a) Analysis of cp38a and cp38b mRNAs in the Ovary.

The poly $(A)^+$ RNA from the ovary (1 μg) was separated by 1% agarose-formamide gel electrophoresis, transferred onto a nylon membrane and fixed. The blot was then hybridized to a ^{32}P-labeled probe generated from three distinct regions (5', 3', and ORF) of cp38a (a) or cp38b (b) cDNA. The membranes were washed at 68℃ in 2×SSC containing 0.5% SDS. Mixed probe of cp38a and cp38b fragments was used to stain both cp38a and cp38b.

(b) Southern Blot Analysis of Carp Genomic DNA.

Carp genomic DNA was digested with *Hin*dIII (lane 1, 3, 5, 7, 9, 11) and *Eco*RI (lane 2, 4, 6, 8, 10, 12), separated by electrophoresis on a 0.8% agarose gel, and transferred onto a nylon membrane. The blot was then hybridized to a ^{32}P-labeled probe generated from three distinct regions (5', 3', and ORF) of cp38a (a) or cp38b (b) cDNA. The membrane was washed under the same condition described in (a). The positions of DNA size markers in kilobases are indicated.

Section 2
Activation of cp38a and cp38b by Environmental Stresses

Activation of p38 is mediated by the upstream MAPKKs, referred to as MKK3 and MKK6,[19-23] whereas JNK is activated by MKK7.[24-29] The targets of active p38 and active JNK seem to be different; p38 phosphorylates MAPKAP kinase-2[17] and JNK phosphorylates c-Jun,[30] although ATF2 is known to be a substrate of both p38 and JNK *in vitro*.[7] Therefore, the p38 and The JNK/SAPK MAP kinase pathways may regulate distinct targets of gene expression. However, in mammalian cells, they appear to be equivalent in an aspect that most of the same stress stimuli trigger both pathways in a similar time course.[7] The fact that MKK4 activates both p38 and JNK might be a reason for this.[19]

In this section, the author examined activation of cp38s by environmental stresses including hypertonic stress in comparison with that of cJNKs.

Materials and Methods

Antibodies

Anti-cp38 antiserum was raised against a peptide encoding residues 325-340 (DQSFESRELDIEEWKR) of cp38a (Fig. 3-4), and recognizes both cp38a and cp38b (data not shown). The Glu (E)-332 residue in the peptide is replaced by Asp (D) in the corresponding sequence of cp38b. The anti-cp38 antibodies were purified by affinity-chromatography on cp38 325-340 peptide-bound TOYOPEARL (AF-Amino TOYOPEARL 650, Tosoh).
Phospho-specific p38 MAPK antibody and anti-ACTIVE JNK pAb were used to stain the active form of cp38s and cJNKs, respectively.

Western Blotting

The cells from the semi-confluent culture of EPC with or without the stress-treatment were lysed in the lysis buffer described in Chapter II-Section 2. The cell lysate containing 1×10^6 cells was resolved by 11% SDS-PAGE, followed by transfer to a PVDF filter (Atto). After blocking in Tris-buffered saline (pH 7.4) containing 0.05% Tween 20 and 5% skimmed milk, the filter was incubated with anti-cp38 antibodies for 1.5 h, and then

washed in Tris-buffered saline (pH 7.4) containing 0.05% Tween 20. A goat anti-rabbit IgG conjugated to horseradish peroxidase (Wako) was used as the secondary antibody. Immunoreactive bands were visualized with ECL detection system (Amersham).

Results and Discussion

Activation of cp38s by Hypertonic Stresses

The author employed Western analysis to detect activation of endogenous cp38s in EPC in response to environmental stress. By using the antibodies specific to the phosphorylated Thr and Tyr residues in the activation regulatory domain of p38, signals could be detected only when cp38s were catalitically activated in the cells, because activation of p38 requires dual-phosphorylation on Thr and Tyr residues by the upstream MAPKK, MKK3 and MKK6.[23]

When EPC was exposed to hypertonic medium supplemented with sorbitol (0.2-1.4 M) or sodium chloride (0.1-0.7 M), the signal intensity increased in a dose dependent manner (Fig. 3-6). Staining with anti-cp38 antibodies indicates that the whole amounts of cp38s in the sample were almost equal (Fig. 3-6). These results suggest that cp38s were phosphorylated and activated in EPC in response to hypertonic stress regardless of osmolyte.

Time Course of cp38 Activation

The author examined when after exposure of EPC to hypertonic stress cp38s exhibits maximal activation. At 5 min after the exposure to 1 M sorbitol, the amount of the active cp38s was most abundant, and a progressive decrease was observed at later times (Fig. 3-7). When EPC was exposed to UV light, cp38s were also activated rapidly within 5 min. But unlike the case of the hypertonic stress, the amount of the active cp38s was sustained at a high level at later times.

In mammalian cells, no significant difference in time course of activation was determined between JNKs and p38s. In response to UV irradiation and treatment with proinflammatory cytokines, both JNK1 and p38α became activated at 30 min after the treatments at the maximal level.[7] When hypertonic stress was given to mammalian cells, JNK1 became

activated more rapidly than p38α.[7)]

In EPC, active cJNKs were detected at the highest level at 15 min after exposure to the hypertonic stress, behind the activation of cp38s (Fig. 3-7). Activation of cJNKs by UV irradiation was also delayed from that of cp38s; it was detected at 5 min at a trace level, rose to the maximal level at 15 min, then and sustained at later times at a high level (Fig. 3-7). These results indicate that there is a 'time-lag' between activation of cp38s and cJNKs in EPC, which differs from mammalian cells. The p38 and The JNK/SAPK MAP kinase pathways seem more apparently to be activated in different time courses and to regulate distinct targets of gene expressions in carp than in mammals. When activation of cJNKs was examined by measuring kinase activity of exogenously expressed cJNKs, the maximal activation was seen at 60 min (in Chapter II-Section 2). The difference in results from the two distinct methods detecting endogenous cJNKs or exogenous cJNKs might be explained by difference in amounts of cJNKs in a cell; exogenous cJNKs were expressed abundantly under the control of CMV promoter, a highly inducible promoter.

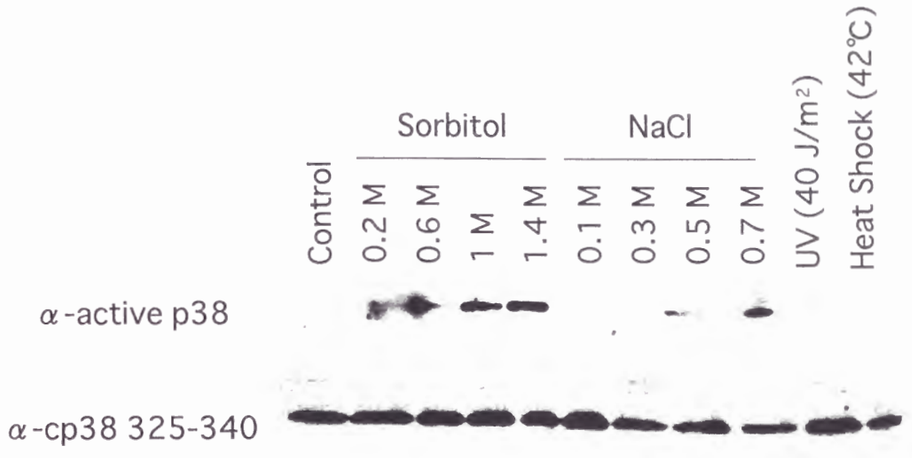

Fig. 3-6 Activation of cp38s by Hypertonic Stress.
EPC was exposed to 0.2-1.4 M sorbitol or 0.1-0.7 M sodium chloride (NaCl) for 30 min. Cells were then lysed, and cell lysates were resolved by SDS-PAGE, blotted , probed with anti-active p38 antibody or anti-cp38 (325-340) antibody, followed by ECL detection.

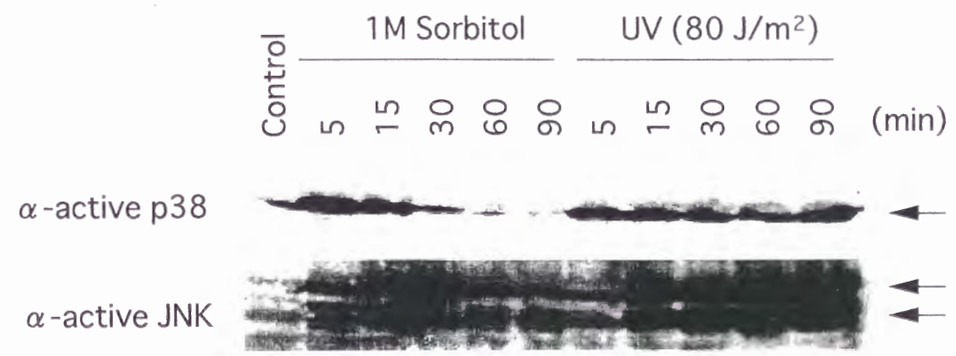

Fig. 3-7 Time Course of cp38 and cJNK Activation.
EPC was exposed to 1M sorbitol or UV light (80 J/m²) for 5-90 min. Cells were then lysed, and cell lysates were resolved by SDS-PAGE, blotted , probed with anti-active p38 antibody or anti-active JNK antibody.

Section 3
Structure of Carp p38 Activator cMKK6 and Its Cytoplasmic Localization Mediated by a Nuclear Export Signal

Recently, Fukuda *et al.*[31] have shown by the analyses on subcellular localization of MAPKK that MAPKK possesses a short peptide sequence showing a nuclear export signal (NES) activity in its N-terminal region. Like the NES which was already identified in HIV-Rev[32] or PKI,[33, 34] this NES was rich in leucine residues, which play a central role for an NES. Cytoplasmic localization of MAPKK by its NES seems to ensure the signal transduction through the classical MAP kinase cascade to proceed in a proper manner, by limiting activation of MAP kinase by MAPKK in the cytoplasm.

The NES sequence has not been found in any MAPKKs participating in the p38 and JNK MAP kinase pathways,[19-29] unlike in MEK (MAPKK).[31, 35] Because at least a part of JNK and p38 is seen to be concentrated in the nucleus following stress stimuli, nuclear translocation of active p38 and JNK probably occurs.[7] However, p38 and JNK can be activated by the corresponding MAPKK in the nucleus as well as in the cytoplasm. It is possible to presume that these stress-activated MAP kinase pathways are regulated under low stringency in terms where in the cell activation of MAP kinase by MAPKK takes place.

In the course of characterizing the stress-activated MAP kinase pathways in carp, the author found that carp MKK6 (cMKK6), an activator of cp38a and cp38b, possessed an NES sequence in its N-terminal region, although no NES-like sequence has been found in MKK3 or MKK6,[19-23] the upstream protein kinases of p38 in other vertebrates. Here the author shows evidence that the NES sequence of cMKK6 functions as a nuclear export signal crucial for cytoplasmic localization of cMKK6. The author also describes that cMKK6 is an upstream protein kinase that phosphorylates cp38a and cp38b.

Materials and Methods

cDNA Cloning

Molecular cloning of the full-length cDNA of cMKK6 was carried out

according to the method described in Chapter II-Section 1. Briefly, a partial sequence of cMKK6 cDNA was obtained by RT-PCR using 5'-ATHATGGCNGTNAARMG-3' (sense) and 5'-CKYTCNGGNGCCATRTA-3' (antisense). The isolated cDNA fragment with significant homology to mammalian MKK6 was used as a probe to screen the carp ovary cDNA library.

Oligonucleotides

Oligonucleotides used as PCR primers for DNA construction are:
cMKK6-5, 5'-CCGCTCGAGCTATGATGGGTCACGCCTGTGG-3';
cMKK6-3, 5'-CGGGATCCGTCCCCGAGGATGCTCTTC-3';
N57s, 5'-TCCCCTCGAGCTATGCGTAAGTGCTCATCCGAT-3';
L46As, 5'-TTCGCATTTAGCCAGCCAGGAT-3';
L46Aa, 5'-ATCCTGGCTGGCTAAATGCGAA-3';
L50As, 5'-CAGCCAGGATGCAGAGCCGCTT-3';
L50Aa, 5'-AAGCGGCTCTGCATCCTGGCTG-3';
L53/55As, 5'-TCTAGAGCCGGCTGCGGCGTCTGACCGT-3';
L53/55Aa, 5'- ACGGTCAGACGCCGCAGCCGGCTCTAGA-3';
N44a, 5'-CCGCTCGAGCTAAATGCGAAAGAGCGAG-3';
cMKK6SEs, 5'-CCTCGTGGATGAAGTGGCGAAG-3';
cMKK6SEa, 5'- CTTCGCCACTTCATCCACGAGG-3';
cMKK6TEs, 5'-AGTGGCGAAGGAAATGGACGCC-3';
cMKK6TEa, 5'- GGCGTCCATTTCCTTCGCCACT-3';
cMKK6KRs, 5'-ATGGCAGTAAGGCGAATCCGG-3';
cMKK6KRa, 5'-CCGGATTCGCCTTACTGCCAT-3'.

DNA Constructs

PCR method was employed to prepare the eucaryotic and the bacterial expression plasmids. The cDNA fragments encoding N-terminal truncated cMKK6 (ΔN57-cMKK6) was amplified with 5' primer N57s, in combination with a 3' primer cMKK6-3. The NES-deleted mutant (Δ45-57-cMKK6) was obtained by ligation of the fragment amplified with cMKK6-5 and N40a primers to the construction of ΔN57-cMKK6 at 5'-end *Xho*I site. The mutagenesis of Leu-46 to alanine and Leu-50 to alanine in cMKK6 was performed by PCR method using mutagenic primers L46As, L46Aa, L50As, and L50Aa in combination with cMKK6-5 and cMKK6-3 to yield

L46A/L50A-cMKK6. To obtain L53A/L55A-cMKK6, mutagenic primers L53/55As and L53/55Aa were used instead of L46As, L46Aa, L50As, and L50Aa. In the same way, SETE-cMKK6 was obtained with mutagenic primers cMKK6SEs, cMKK6SEa, cMKK6TEs, and cMKK6TEa, and KR-cMKK6 was obtained with cMKK6KRs and cMKK6KRa primers. *Xho*I and *Bam*HI sites at 5' and 3' ends of PCR products were used to clone the mutants of cMKK6 and wild-type cMKK6 (WT-cMKK6) into pcDNA3.1A-Myc-His.

Transfection

Cos-7 (originated from African green monkey) was seeded in 24-well plates in Dulbecco's modified Eagle's medium (DMEM) supplemented 10% FBS and 10 mM HEPES for 16 h before transfection. The total amount of DNA in the transfection was 0.75 μg per well. The plasmids were mixed with lipofectamine (Gibco-BRL) in serum-free Opti-MEM (Gibco-BRL) and cells were transfected. Five hours after transfection, the cells were incubated in medium containing 10% FBS for an additional 16-40 h.

Cell Staining

The cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 30 min, and then permeabilized with 0.4% Triton X-100 in PBS for 10 min at room temperature. After blocking with 10% goat serum in PBS, the coverslips were incubated with primary antibodies at room temperature for 1 h, and then with the appropriate secondary antibodies (goat IgG).

Conjugation of Synthetic Peptides to Ovalbumin

A peptide (L peptide) corresponding to the sequence of residues 45-57 of cMKK6 (CLLSQDLEPLALSD) and its mutant peptide (A peptide) (CLASQDAEPAAASD) in which four leucines (residues 46, 50, 53, and 55) were replaced by alanines were synthesized. These synthetic peptides were conjugated to ovalbumin (OV, Sigma) with a bifunctional cross-linking reagent sulfo-SMCC (Pierce). Briefly, sulfo-SMCC-activated OV was prepared by incubating OV (5 mg/ml) and sulfo-SMCC (8 mg/ml) in PBS (100 mM NaH_2PO_4, pH 8.0, 150 mM NaCl) at 20℃ for 1 h. Excess cross-linker was removed by a prepacked gel filtration column, Bio-Gel-10 DG

(Bio-Rad), in PBS (pH 7.0). The L peptide (3 mg/ml) or the A peptide (3 mg/ml) was added to the sulfo-SMCC-activated OV (~2 mg/ml). After incubation for 3 h at room temperature, free peptide was removed by a prepared gel filtration column, Bio-Gel-10 DG (Bio-Rad), equilibrated in PBS (pH 7.0). The L peptide-conjugated OV (L-OV) or the A peptide-conjugated OV (A-OV) was concentrated by Ms. BTAURY-KN (Atto). The coupling ratios of both conjugations were ~10-15 peptides/OV molecule because the L-OV and the A-OV had an apparent molecular mass of ~65 kDa as estimated by SDS-polyacrylamide gel electrophoresis (data not shown).

Microinjection

Rat 3Y1 cells were plated onto CELLocate coverslips (Eppendorf) and cultured in DMEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 0.2 mg/ml kanamycin) for 2 days, and then placed in starvation medium (DMEM without FBS) for 24-48 h before microinjection. Microinjection was performed using an IM-188 microinjection apparatus (Narishige). Cells cultured on marked areas of CELLocate coverslips were microinjected with the samples.

Results and Discussion

cDNA Cloning of cMKK6 and Its Expression in Tissues

PCR cloning of carp MKK6 cDNA fragment successfully yielded an expected size of DNA corresponding to a potential MKK3 or MKK6. In fact, a homology search with the GenBank database revealed that the cDNA fragment was homologous to the inner part of human MKK3 and MKK6 cDNAs (data not shown). By using this cDNA fragment as a probe, the author screened the carp ovary cDNA library. Screening of approximately 3×10^5 plaques yielded 11 positive clones. The longest clone was subjected to determination of the complete nucleotide sequence. The in-frame stop codons in the 5' and 3' regions of the cDNA indicated that this clone contains the entire cMKK6 coding region. The amino acid sequences predicted by the nucleotide sequence of cMKK6 (Fig. 3-8) contained 404 residues with a predicted mass of 45.4 kDa.

Multiple alignments of amino acid sequence of cMKK6 with those of

human MKK3 and MKK6 are shown in Fig. 3-9. Comparison with the protein sequences by m-align indicated that cMKK6 is 37%, 37%, 83%, 46%, 87%, and 43% identical in primary structure to human MEK1,[36] MEK2,[36] MKK3,[19-23] MKK4,[19] MKK6,[19-23] and MKK7,[24-29] respectively. MAPKKs are known to be activated by MAPKKK through phosphorylation of adjacent Ser (S) and Thr (T) (or Ser (S)) residues.[23] These putative phosphorylation sites of cMKK6 are Ser-277 and Thr-281, possessing the Thr-Val-Ala-Lys-Tyr motif, which is conserved in p38 activators, MKK3 and MKK6 (Fig. 3-9). cMKK6 is most homologous to human MKK6 in overall structure. It is assumed that cMKK6 is an activator for cp38a and cp38b, participating in the p38 MAP kinase pathway. Northern blot analysis revealed that cMKK6 mRNA was expressed abundantly in the ovary (Fig. 3-10). In addition to 3.6 kb mRNA which was commonly detected in all the tissues examined, two smaller mRNAs (3 kb and 2 kb) were expressed exclusively in the ovary and exhibited stronger signals than the 3.6 kb mRNA (Fig. 3-10). Exclusive expression of cMKK6 mRNA together with cp38a and cp38b mRNAs in the ovary implies some important role(s) of the p38 MAP kinase pathway in the ovary of carp as discussed in Chapter II.

Cytoplasmic Localization of cMKK6 Mediated by a Nuclear Export Signal

The author found an important difference in primary structure between cMKK6 and human MKK3 or MKK6; cMKK6 possesses an NES-like sequence in its N-terminal region like MAPKKs (human MEK1, MEK2 , and frog MAPKK, participants in the classical MAP kinase pathway),[31, 36] but unlike other MAPKKs (MKK3, MKK4, MKK6, and MKK7, participants in the stress-activated (p38 and JNK) MAP kinase pathway).[19-29] The positions of four leucine residues in the sequence of residues 46-55 of cMKK6 are completely conserved in the sequences of residues 33-42 of human MEK1 and are similar to those of residues 37-46 of PKIα and residues 73-84 of Rev (Fig. 3-11). In order to test whether the NES-like sequence in cMKK6 acts as an NES to ensure cytoplasmic localization of cMKK6, several plasmids harboring various mutants of cMKK6 and the wild-type cMKK6 were constructed and transfected into Cos-7 (Fig. 3-12). The subcellular localization of exogenously expressed proteins which were Myc epitope-tagged in the C-terminus was determined by immunological cell-staining with anti-Myc antibody.

First, the author examined the subcellular distribution of cMKK6. WT-cMKK6 was located only in the cytoplasm and was almost completely excluded from the nucleus in spite of its relatively small size (~48 kDa), which could enter the nucleus by diffusion (Fig. 3-13).[37, 38] Likewise, an active form SETE-cMKK6 (Ser-277 and Thr-281 are replaced by glutamine) and a dominant-negative form KR-cMKK6 (Lys-152 is replaced by arginine) were seen only in the cytoplasm (Fig. 3-13). These results suggest that the cytoplasmic localization of cMKK6 is regulated independently of its kinase activity.

In order to know whether the leucine-rich sequence (45-57) in N-terminal region is necessary for the cytoplasmic localization of cMKK6, the author examined the subcellular distribution of ΔN57-cMKK6 and Δ45-57-cMKK6. As a result, ΔN57-cMKK6 was located in both the nucleus and the cytoplasm (Fig. 3-13). Interestingly, Δ45-57-cMKK6 was concentrated to the nucleus, although the reason was not known at this time (Fig. 3-13). The N-terminal sequence of residues 45-57 may work to locate cMKK6 in the cytoplasm.

Recently, it has shown that the NES of PKIα or MAPKK is autonomous enough to trigger nuclear export when chemically conjugated to the transport substrate.[31, 33] To test whether the leucine-rich sequence of cMKK6 can act as an autonomous NES, the peptide corresponding to residues 45-57 of cMKK6 (the L peptide) was synthesized and chemically conjugated to ovalbumin (OV) through an additional N-terminal cysteine of the peptide. The resultant L-OV had an apparent molecular mass of ~65 kDa and was estimated to have ~10-15 L peptides per OV molecule. When injected into the nucleus, L-OV was found in the cytoplasm and was excluded from the nucleus abundantly within 20 min. In contrast, co-injected RITC-BSA remained in the nucleus (Fig. 3-14). The leucines in the NES of PKIα and MAPKK were shown to be important for the NES activity.[31, 33] Then, the mutant peptide in which all the four conservative leucines (Leu-46, Leu-50, Leu-53, and Leu-55 of cMKK6) were replaced by alanine (the A peptide) was synthesized and conjugated to OV. The resultant A-OV also had an apparent molecular mass of ~65 kDa. When injected in the nucleus, A-OV was incapable of crossing the nuclear envelope and remained in the nucleus (Fig. 3-14). These results indicate that the N-terminal sequence of residues 45-57 (L peptide) of cMKK6 is an autonomous NES and that the

four leucines in the sequence are crucial.

In order to confirm the importance of the NES of cMKK6 in its subcellular distribution, leucines in the NES sequence of cMKK6 were replaced by alanine. Two mutant cMKK6s of double mutation were produced: L46A/L50A-cMKK6 and L53A/L55A-cMKK6. A plasmid each mutant cMKK6 was transfected into Cos-7 and the subcellular distribution of the exogenous protein (L46A/L50A-cMKK6 or L53A/L55A-cMKK6) was determined. Whereas wild-type cMKK6 was localized exclusively in the cytoplasm, both of the mutant cMKK6s were distributed evenly throughout the cell, present in both the nucleus and the cytoplasm (Fig. 3-15). These results may be interpreted as suggesting that cMKK6 could cross the nuclear envelope to the nucleus by diffusion if it did not have NES and that the NES of cMKK6 may thus define the cytoplasmic localization of cMKK6. Furthermore, the data shown in Fig. 3-15 support the importance of the four leucine residues in the NES of cMKK6.

The NES of cMKK6 would be used to ensure permanent cytoplasmic localization of cMKK6. As cMKK6 must receive the signal from the upstream kinase MAPKKK in the cytoplasm, the existence of this mechanism for ensuring cytoplasmic localization of cMKK6 by an active process would be reasonable. The cytoplasmic localization of cMKK6 would be important for the proper, regulated signal transduction of stress-response, which needs the activation of cp38a and cp38b in the cytoplasm. In terms that the NES exists in cMKK6, an activator of cp38s, but not in any activators of p38s of other animals, the p38 MAP kinase pathway would be precisely regulated in carp compared with those in other animals. Differences in activation patterns of cJNKs and cp38s might be explained by the remarkable difference in N-terminal structures of the upstream activators, cMKK4 and cMKK6; cMKK6 has an NES, which defines its cytoplasmic localization and thereby regulates the proper activation of cp38s by cMKK6 in the cytoplasm, whereas cMKK4 does not have an NES (unpublished data), and thus can stay in the nucleus, where it cannot receive a signal from the membrane until it comes out from the nucleus by diffusion.


```
-444       GCAGGAAGAACGTGTGTTAGCAGGGGCTCACACTGATCCAAGTTAGGAGCTCTCAGCTGCTGGCCTTGGCCCATCATGTAAGTG  -361
-360 CCTGCCGAGGTTTCTTTGCAGAGCGGTTGCAGAAATCTCTCCGTTTTCGTTGCGTGCAATTGAAAGGCCAGCCTTTGCACGGAGAACAAG  -271
-270 TTACCCGACGCCCAAAGGCTCCGAGTGCATGAAGATCGCGGCTTGTATTTTGCACCTTTGTTGCAAGCAAGCCCGCTTGAAGCCTCACTT  -181
-180 CGAGGGATAGATGGAAGGAGGGAGCGACAAGGAAAGCAAAGTCTTTTGTGCTTCCCCTTCCCCCAACCCAAAAGAGGAGATGTCTCTGCC   -91
 -90 AAAAGGAGACTGGAATGAGTCAGTCGGGTGGGTGTGGGCTGCTCATGTGCCATCAGGTATGTGGATTGCCCCACTCTGGTGTCACTCACA    -1

   1 ATGATGGGTCACGCCTGTGGCAGCAGCGCCTCCGATCTGCTTCAGATGGAGAGAACGTACCTGGCCCTGCTGTCTCGCCTCCCTCTTTCA    90
   1 M  M  G  H  A  C  G  S  S  A  S  D  L  L  Q  M  E  R  T  Y  L  A  L  L  S  R  L  P  L  S    30

  91 CACTTGCACTCTCTCTCCTCGCACTCTCTCGCTCTTTCGCATTTACTCAGCCAGGATCTAGAGCCGCTTGCGCTGTCTGACCGTAAGTGC   180
  31 H  L  H  S  L  S  S  H  S  L  A  L  S  H  L  L  S  Q  D  L  E  P  L  A  L  S  D  R  K  C    60
 181 TCATCCGATAGGGAATCTCGCAATTCCATGGGGAGCAGAAATAAAGCAGGTAAAAAGAAGCCACCACTCAAGCTTCCGAAAGAGGTGTTT   270

  61 S  S  D  R  E  S  R  N  S  M  G  S  R  N  K  A  G  K  K  K  P  P  L  K  L  P  K  E  V  F    90
 271 GAGAAACCCCAACCTGCTCCTACACCCCCGAGAGACCTGGACTCCAAAGCCTGTGTTACTATTGGAGATAAGAACTTTGTGGTGAAGGCC   360
  91 E  K  P  Q  P  A  P  T  P  P  R  D  L  D  S  K  A  C  V  T  I  G  D  K  N  F  V  V  K  A   120

 361 GATGATTTGGAGCAGATTGGAGAGTTGGGGCGAGGGGCGTATGGAGTGGTGGACAAGATGAGACACGTCCCAAGTGGCGTAATAATGGCA   450
 121 D  D  L  E  Q  I  G  E  L  G  R  G  A  Y  G  V  V  D  K  M  R  H  V  P  S  G  V  I  M  A   150

 451 GTAAAGCGAATCCGGGCCACAGTAAACACACAGGAGCAGAAACGGCTGCTAATGGATCTGGACATCTCCATGAGAACAGTCGACTGCTTT   540
 151 V  K  R  I  R  A  T  V  N  T  Q  E  Q  K  R  L  L  M  D  L  D  I  S  M  R  T  V  D  C  F   180

 541 TATACTGTTACCTTCTATGGAGCCCTGTTCAGAGAGGGTGACGTGTGGATCTGCATGGAGCTGATGGACACCTCTCTGGATAAATTTTAT   630
 181 Y  T  V  T  F  Y  G  A  L  F  R  E  G  D  V  W  I  C  M  E  L  M  D  T  S  L  D  K  F  Y   210

 631 AAACAGGTGCATGAGAAGGGTATGACCATCCCAGAGGACATCCTGGGAAAGATCACAGTTTCTATCGTGAAAGCATTGGAGCATCTCCAC   720
 211 K  Q  V  H  E  K  G  M  T  I  P  E  D  I  L  G  K  I  T  V  S  I  V  K  A  L  E  H  L  H   240

 721 AGCAACCTGTCAGTGATACACAGAGATGTGAAACCCTCTAACGTCCTGATAAACATGCAGGGTCAGGTGAAAATGTGTGATTTTGGCATC   810
 241 S  N  L  S  V  I  H  R  D  V  K  P  S  N  V  L  I  N  M  Q  G  Q  V  K  M  C  D  F  G  I   270

 811 AGCGGGTACCTCGTGGATTCAGTGGCGAAGACAATGGACGCCGGCTGCAAGCCATACATGGCGCCTGAGAGAATCAATCCAGAGACCAAT   900
 271 S  G  Y  L  V  D  S  V  A  K  T  M  D  A  G  C  K  P  Y  M  A  P  E  R  I  N  P  E  T  N   300

 901 CAGAAAGGCTACAATGTCAAGTCTGATATCTGGAGTTTAGGAATCACAATGATCGAGCTGGCCATTCTGCGGTTTCCCTATGACTCATGG   990
 301 Q  K  G  Y  N  V  K  S  D  I  W  S  L  G  I  T  M  I  E  L  A  I  L  R  F  P  Y  D  S  W   330

 991 GGAACGCCATTTCAGCAGCTCAAGCAGGTGGTGGAAGAGCCGTCGCCCCAGCTGCCTGCAGACCGCTTCTCACCCGAGTTTGTGGACTTC  1080
 331 G  T  P  F  Q  Q  L  K  Q  V  V  E  E  P  S  P  Q  L  P  A  D  R  F  S  P  E  F  V  D  F   360

1081 ACGTCACAATGCTTAAGGAAGAATTCCAAAGAGCGGCCGACTTACACAGAACTAATGCAACATCCCTTTTTCACCATCCATGACTCCAAA  1170
 361 T  S  Q  C  L  R  K  N  S  K  E  R  P  T  Y  T  E  L  M  Q  H  P  F  F  T  I  H  D  S  K   390

1171 GACACCGACGTCGCTAGCTTTGTGAAGAGCATCCTCGGGGACTGAGAACGTCCCTCTCTGCAAAACTGACGTGTTGCCATGGGGGGAGAC  1260
 391 D  T  D  V  A  S  F  V  K  S  I  L  G  D  *                                                404

1261 GGGACTATTTGAGAAAAAGCACCAATAGCAAGACTAACACCTGTAAGACACGCCCACCTCAGCAGCCAACAGTGCACAAGCACTCAGGAG  1350
1351 GGTCTGGGGGAAAAAAACACAGAAACTGAGGGTGGGCAGGCCGTTTCCTAGCAAAACACGGAGTAGGGCATGGGACGGGAATCTCAGCAC  1440
1441 GTGTGTGCATGTGTGGATGCGTGTGCGAGAATGAGAGTGTGTGTGTATGTGTGTGTGAATTCTTCGCCTGCCTGTATGTGCATACATGTG  1530
1531 CATTAAAAGGTTTAACAGTCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA                                      1583
```

Fig. 3-8 Nucleotide and Deduced Amino Acid Sequences of cMKK6.
The open reading frame (404 amino acids) is preceded by four in-frame stop codons, indicated by underlining. The putative phosphorylation site is double-underlined. The predicted stop codon is denoted by an asterisk.

```
cMKK6     MMGHACGSSASDLLQMERTYLALLSRLPLSHLHSLSSHSLALSHLLSQDLEPLALSDRKC   60
hMKK3     M-------------------------------------------------------------   1
hMKK6     M-------------------------------------------------------------   1
          *

cMKK6     SSDRESRNSMGSRNKAGKKKPPLKLPKEVFEKPQPAPTPPRDLDSKACVTIGDKNFVVKA  120
hMKK3     ----------------------------SKPPAPNPTPPRNLDSRTFITIGDRNFEVEA   32
hMKK6     -----------SQSKGKKRNPGLKIPKEAFEQPQTSSTPPRDLDSKACISIGNQNFEVKA   50
                                      *    **** ***     **  ** * *

cMKK6     DDLEQIGELGRGAYGVVDKMRHVPSGVIMAVKRIRATVNTQEQKRLLMDLDISMRTVDCF  180
hMKK3     DDLVTISELGRGAYGVVEKVRHAQSGTIMAVKRIRATVNSQEQKRLLMDLDINMRTVDCF   92
hMKK6     DDLEPIMELGRGAYGVVEKMRHVPSGQIMAVKRIRATVNSQEQKRLLMDLDISMRTVDCP  110
          ***  * ********** * **  ** ************ ************ ******

cMKK6     YTVTFYGALFREGDVWICMELMDTSLDKFYKQVHEKGMTIPEDILGKITVSIVKALEHLH  240
hMKK3     YTVTFYGALFREGDVWICMELMDTSLDKFYRKVLDKNMTIPEDILGEIAVSIVRALEHLH  152
hMKK6     FTVTFYGALFREGDVWICMELMDTSLDKFYKQVIDKGQTIPEDILGKIAVSIVKALEHLH  170
           ****************************  *  *  ******** * **** ******

cMKK6     SNLSVIHRDVKPSNVLINMQGQVKMCDFGISGYLVDSVAKTMDAGCKPYMAPERINPETN  300
hMKK3     SKLSVIHRDVKPSNVLINKEGHVKMCDFGISGYLVDSVAKTMDAGCKPYMAPERINPELN  212
hMKK6     SKLSVIHRDVKPSNVLINALGQVKMCDFGISGYLVDSVAKTIDAGCKPYMAPERINPELN  230
          * ****************  * ******************* ***************** *

cMKK6     QKGYNVKSDIWSLGITMIELAILRFPYDSWGTPFQQLKQVVEEPSPQLPADRFSPEFVDF  360
hMKK3     QKGYNVKSDVWSLGITMIEMAILRFPYESWGTPFQQLKQVVEEPSPQLPADRFSPEFVDF  272
hMKK6     QKGYSVKSDIWSLGITMIELAILRFPYDSWGTPFQQLKQVVEEPSPQLPADKFSAEFVDF  290
          **** **** ********* ******* ********************** ** *****

cMKK6     TSQCLRKNSKERPTYTELMQHPFFTIHDSKDTDVASFVKSILGD--                404
hMKK3     TAQCLRKNPAERMSYLELMEHPFFTLHKTKKTDIAAFVKKILGEDS                318
hMKK6     TSQCLKKNSKERPTYPELMQHPFFTLHESKGTDVASFVKLILGD--                334
          * *** **  **  * *** ***** *  * ** * *** ***
```

Fig. 3-9 Comparison of Amino Acid Sequences of cMKK6 with Human MKK3 and MKK6.

The sequences comprise the result in this study, and those of human MKK3 and MKK6.[19-23] Identical residues are indicated by asterisks, and gaps introduced into the sequences are depicted by hyphens. The positions of the conservative leucine residues are indicated by shadowing.

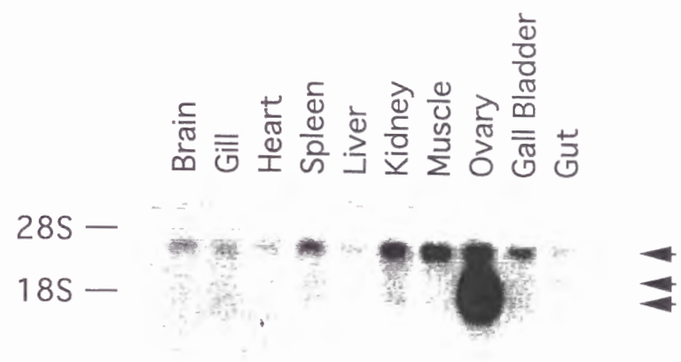

Fig. 3-10 Tissue Distribution of Carp MKK6 mRNA.

Poly $(A)^+$ RNA samples were prepared from the brain, gill, heart, spleen, liver, kidney, muscle, ovary, gall bladder, and gut. Each RNA sample (1 μg) was separated by 1% agarose-formaldehyde gel electrophoresis, transferred onto a nylon membrane and fixed. The cDNA fragment derived by RT-PCR cloning was used as a probe. After hybridization, the membrane was washed at 65℃ in 2×SSC containing 0.5% SDS. The positions of 28S and 18S rRNAs are indicated on the left.

cMKK6	45-57	LLSQDLEPLALSD
cMEK2	33-45	ALQRKLGELDLDE
hMEK1	32-44	ALQKKLEELELDE
PKI α	37-46	LALKLAGLDI
Rev	73-84	LQLPPLERLTL

Fig. 3-11 Comparison of the NES Sequences.

The conservative hydrophobic residues (leucine and isoleucine) in the NES sequence are shadowed. cMEK2 is carp homologue of human MEK2. hMEK1 stands for human MEK1.[36]

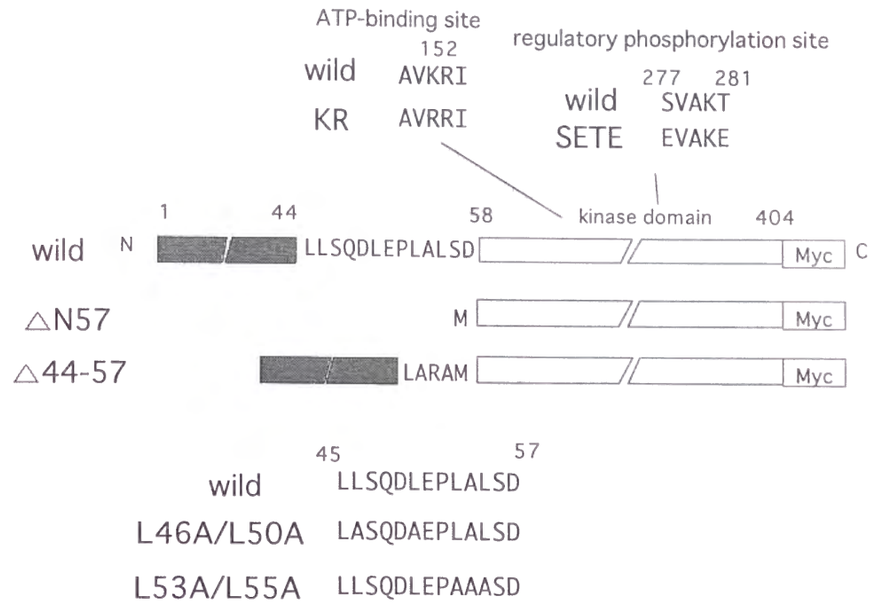

Fig. 3-12 Constructions of Mutants of cMKK6.

To obtain constitutively active cMKK6, Ser-277 and Thr-281 were replaced by glutamines (SETE). To obtain dominant negative cMKK6, Lys-152 was replaced by arginine (KR). N-terminal region containing the NES sequence was truncated by removing residues 1-57 (replaced by methionine)(ΔN57). The NES was deleted by replacement with LARAM sequence (Δ45-57).

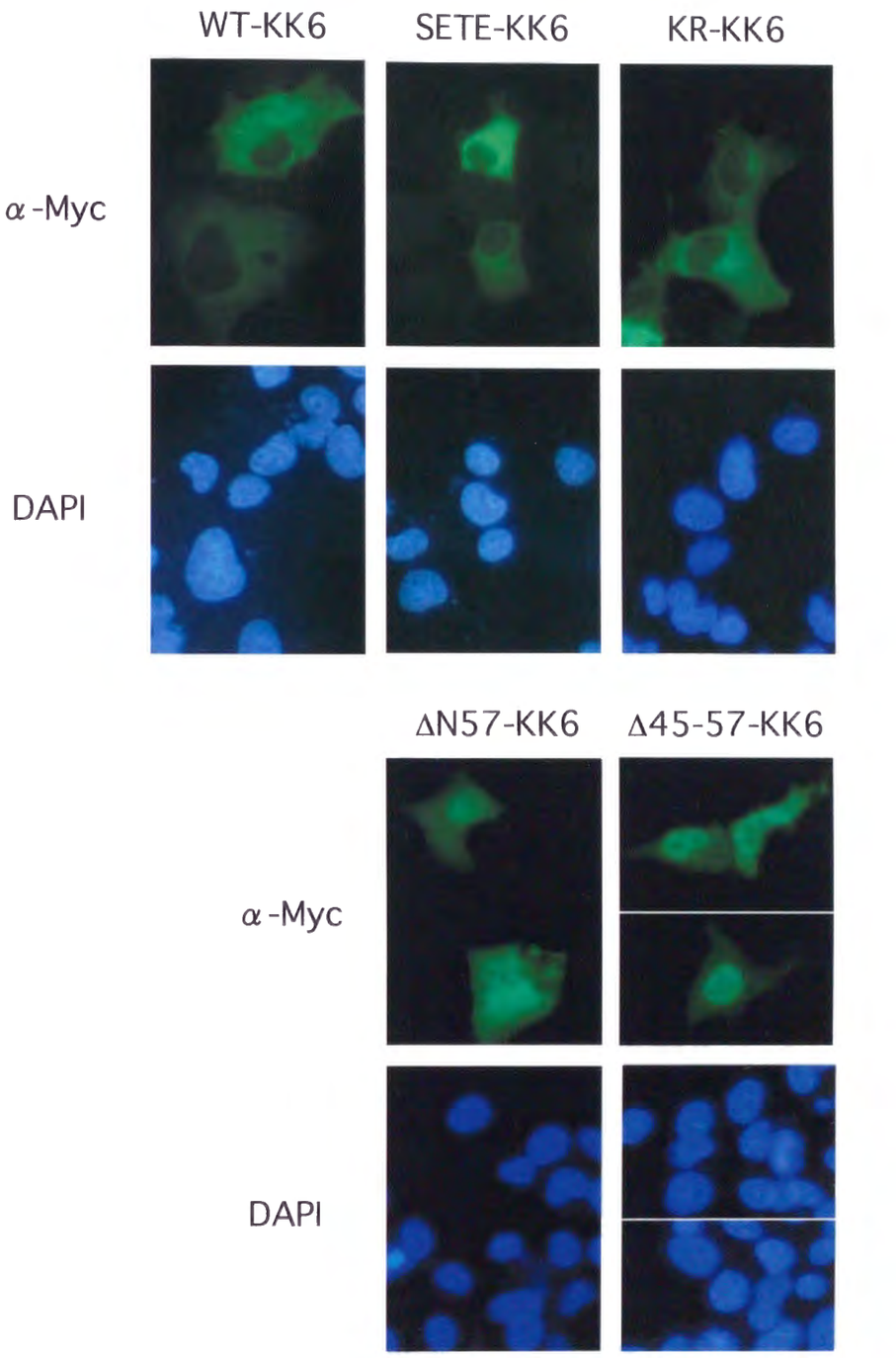
WT-KK6
SETE-KK6
KR-KK6
α-Myc
DAPI
ΔN57-KK6
Δ45-57-KK6
α-Myc
DAPI

Fig. 3-13 The N-terminal Region is Necessary for Cytoplasmic Localization of cMKK6.

Cos-7 was transfected with a plasmid expressing Myc-tagged protein of wild-type cMKK6 (WT-KK6), SETE-cMKK6 (SETE-KK6), KR-cMKK6 (KR-KK6), ΔN57-cMKK6 (ΔN57-KK6), or Δ45-57-cMKK6 (Δ45-57-KK6). Cells were stained with anti-Myc antibody. The positions of the nucleus are shown by DAPI staining.

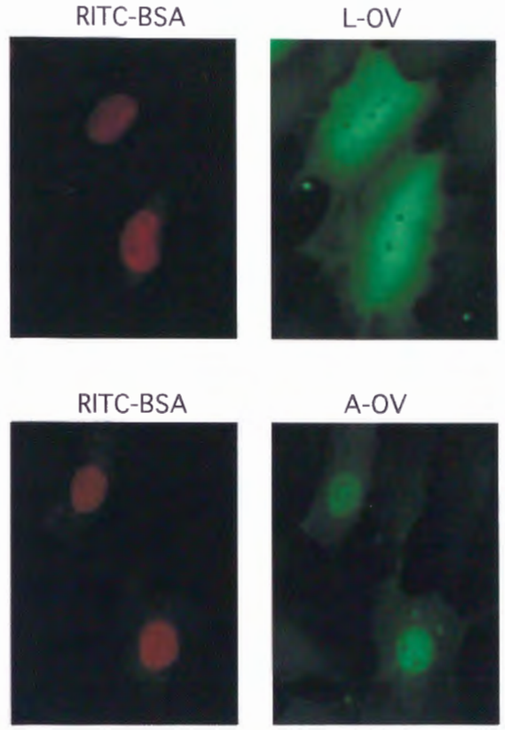

Fig. 3-14 A Synthetic Peptide Corresponding to the Sequence of Residues 45-57 of cMKK6 is Able to Direct OV from the Nucleus to the Cytoplasm.

A mixture of L-OV (1.2 mg/ml) and RITC-BSA (1.0 mg/ml) was injected into the nuclei of 3Y1 cells (upper). Cells were fixed 20 min after injection and stained with anti-OV antibody. A mixture of A-OV (1.2 mg/ml) and RITC-BSA (1.0 mg/ml) was injected into the nuclei and processed as above (lower).

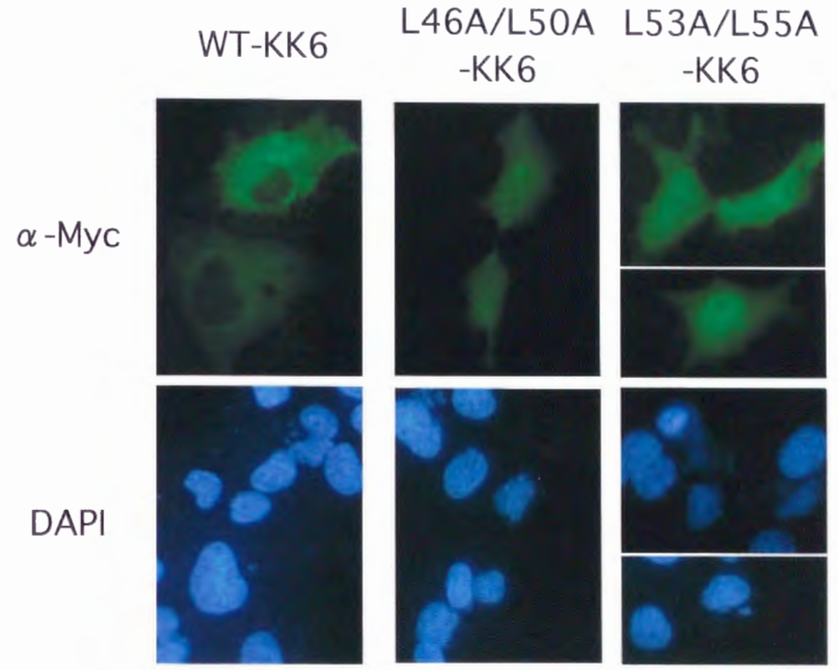

Fig. 3-15 Cytoplasmic Localization of cMKK6 Requires Its Leucine-rich NES Sequence.

Cos-7 was transfected with a plasmid expressing either wild-type cMKK6 (WT-KK6), L46A/L50A-cMKK6 (L46A/L50A-KK6), or L53A/L57A-cMKK6 (L53A/L55A-KK6). Cells were stained with anti-Myc antibody. The positions of the nucleus are shown by DAPI staining.

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SUMMARY AND CONCLUSION

(1) The author revealed that fish cell lines EPC, EK-1, and BF-2 grew at high rate in the hypotonic medium. The author also revealed that EPC and EK-1 seemed to be sensitive to the hypertonicity for a short term period but adapted to the hypertonic medium, resuming cell growth after a long term period. These features of the fish cell lines were not observed in mammalian cell lines examined in this study. The high adaptability of the fish cell lines, BF-2, EK-1, and EPC, to different osmolarities raises a requirement for a further study on the osmotic-responsive mechanism in fish cells necessary for the adaptation.

The author also investigated the effects of osmotic stresses on apoptotic cell death of a fish cell line EPC. EPC showed DNA fragmentation, which is a biochemical feature of apoptosis, under hypertonic conditions, in 400-600 mOsm/kg media with sodium chloride supplementation. Similar results were obtained upon exposure to 450 mOsm/kg medium with sorbitol. DNA fragmentation increased significantly within 3 h after exposure. Nuclear condensation, which is a morphological hallmark of apoptosis, was also observed in the culture of EPC exposed to hypertonic stress. The amount of native nucleosomal DNA was evaluated to know whether the whole cell population undergoes apoptosis. As a result, hypertonicity below 500 mOsm/kg triggered apoptotic cell death only in a part of the whole cell population, while 600 mOsm/kg brought about cell death in a large proportion of the population by necrosis as well as apoptosis. In contrast, hypotonic media (150 and 200 mOsm/kg) did not induce DNA fragmentation. DNA fragmentation of EPC induced by hypertonic stress was suppressed in the presence of Zn^{2+}, suggesting that a Zn^{2+}-susceptible endonuclease(s) may be responsible for cleavage of nucleosomal DNA.

Effects of osmotic acclimatization on cell death of EPC were investigated. The culture acclimatized to moderate hypertonic medium (400 mOsm/kg) showed less DNA fragmentation with the exposure to severe hypertonic media (500 and 600 mOsm/kg), when compared to the culture acclimatized to isotonic medium (300 mOsm/kg). In contrast, the culture acclimatized to moderate hypotonic medium (200 mOsm/kg) exhibited more remarkable DNA fragmentation. The suppression of apoptosis by hypertonic acclimatization could be explained by the shift of the threshold of apoptosis

in osmotic pressure. Because DNA fragmentation with the exposure to hypotonic medium was not observed in all the cultures acclimatized to 200, 300, or 400 mOsm/kg medium, EPC has an intrinsic tolerance against hypotonic environment.

(2) The author attempted to characterize the stress-activated signal transduction pathways, which seem to be necessary to initiate adaptations to a stress-environment such as hypertonicity by regulating the levels of gene products. Two major signal transduction pathways, the JNK/SAPK and the p38 MAP kinase pathways, known to be activated in response to hypertonic stress in mammalian cells were investigated using carp as a material.

The author first isolated two distinct stress-activated protein kinase (cJNKa and cJNKb) cDNAs from a carp ovary cDNA library. These cDNAs contained a full-length open reading frame encoding 427 amino acid residues with a predicted mass of 48.6 kDa. The deduced amino acid sequences of cJNKa and cJNKb were 95.8% identical and showed a high degree of sequence similarity to mammalian JNK/SAPK subgroup. By Northern blot analysis, cJNKs were found to be abundant in the brain and ovary. Detailed study by RT-PCR assay revealed ubiquitous expression of JNKb, although expression of JNKa was specific to the brain and ovary. The high level expression of both JNKa and b in the ovary implies that JNKs play an important role in egg maturation or ectogenetic early development.

Activation of cJNK and cJNKb in EPC was examined by measuring the kinase activity of exogenously-expressed cJNKa or cJNKb. Both cJNKa and cJNKb were activated in EPC in response to hypertonic stress, UV irradiation, and heat shock. No apparent differences in activation pattern were observed between cJNKa and cJNKb in this study. Both of them appear to function in EPC as a signal transducer for environmental stress.

The author next characterized the p38 MAP kinase pathway. Two distinct p38 (cp38a and cp38b) cDNAs were isolated from the ovary cDNA library. These cDNAs contained a full-length open reading frame encoding 361 amino acid residues with a predicted mass of 41.7 kDa (cp38a) or 41.5 kDa (cp38b). The deduced amino acid sequences of cp38a and cp38b both showed the highest homology to human p38α. cp38 mRNA was predominantly expressed in the ovary, whereas expression of cp38b mRNA was ubiquitous. Immunoblot analysis detecting specifically the activated

p38 revealed that cp38s (cp38a and/or cp38b) were activated in response to hypertonic stress. Interestingly, activation of cp38s was seen to be earlier than that of cJNKs in EPC, although no significant difference in activation-time course has not been observed between p38 and JNK in mammalian cells. The p38 MAP kinase pathway might function in a different manner from The JNK/SAPK MAP kinase pathway, although they are activated in response to the same environmental stress.

An upstream activator of cp38s, termed cMKK6 (carp MAPKK homologous to human MKK6) was identified. The cDNA of cMKK6 was isolated from the ovary library. The deduced amino acid sequence contained 404 amino acid residues with a predicted mass of 45.4 kDa and showed 87% homology to human MKK6. cMKK6 mRNA was also expressed abundantly in the ovary. The author found an important difference in primary structure between cMKK6 and other stress-activated MAPKKs; cMKK6 possessed an NES-like sequence in its N-terminal region (residues 45-57). The author proved that the NES-like sequence functioned as an NES in Cos-7 (also in RBCF1, a goldfish fin cell line), and was necessary and sufficient for an NES activity to ensure cytoplasmic localization of cMKK6. The existence of the NES in cMKK6 implies that the p38 MAP kinase pathway would be precisely regulated in carp compared with those in other animals.

The distinct features of the two stress-activated signal transduction pathways (the p38 and The JNK/SAPK MAP kinase pathways) and the precise regulation of the p38 MAP kinase pathway by ensuring cytoplasmic localization of MAPKK (cMKK6) have been possibly retained in fish through molecular evolution, since they are important for fish to survive against environmental stresses, particularly in the ectogenetic early development.

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3) Hisashi Hashimoto, Yoshiyuki Matsuo, Kazuharu Takeuchi, Yoshihiro Yokoyama, Haruhiko Toyohara, and Morihiko Sakaguchi: Suppression of apoptosis by acclimatization of fish cells to hypertonicity., in preparation.

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6) Hisashi Hashimoto, Haruhiko Toyohara, Yoshihiro Yokoyama, Morihiko Sakaguchi, Kenjiro Ozato, and Yuko Wakamatsu: Effects of carp (*Cyprinus carpio* L.) serum on the growth of goldfish (*Carassius auratus* L.) fin cells in early passage. *J. Fish Biol.*, 50, 201-207 (1997).

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